Oscillating magnet array-based nanomagnetic gene transfection of mammalian cells relevant to regenerative medicine

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Abstract

Successful gene therapy depends upon specific gene delivery into the cells and tissues of interest. Nanomagnetic gene transfection is a relatively new gene delivery technique that has attempted to meet that need and has been effectively used with both viral and non-viral vector systems.

It uses magnetic nanoparticles (MNPs) in assisting and directing specific delivery of reporter or therapeutic genes on a single cell basis, in the presence of an externally introduced oscillating magnet. The novelty of the lateral oscillation further stimulates endocytosis of MNP:plasmid complexes with improved *in vitro* transfection efficiency compared to the static magnet application and other non-viral gene delivery approaches. This work's purpose was to contribute in the optimisation of this tool for safe and efficient gene delivery, and to investigate the applicability of the method in a wider range of cell types used for regenerative medicine purposes, improving transfection efficiency and duration. Novel transfection experiments using commercially available MNPs coupled to a reporter gene, demonstrated higher levels of transfection efficiency (differing among cell types) and cell viability (80-94%), at the lowest reagent concentrations across all post-transfected cell types, compared with the most widely used cationic lipids (Lipofectamine) and/or electroporation.

In particular, using human lung mucoepidermoid carcinoma cells (NCI-H292), the magnetic field requirements for transfection were evaluated; using human osteosarcoma fibroblasts (MG-63), a nanomagnetic transfection protocol at shorter transfection times (30 min) was established for increased transfection efficiency (53% oscillating and 49% static

magnet, 7% at 30 min and 24% at 6 hr Lipofectamine, and 21% electroporation); using mouse embryonic fibroblasts (NIH-3T3), the 30 min-protocol was applied further (25% oscillating and 22% static magnet, 2% at 30 min and 22% at 6 hr Lipofectamine); using human mesenchymal stem cells (hMSCs), the ability of the method to transfect primary cells and to retain key markers for multipotency was demonstrated; using human embryonic stem cells (hESCs), the transfection capability in additional types of primary cells was shown, along with indications of retention of key markers for pluripotency and differentiation, although further work is required to confirm this finding.

Nanomagnetic gene transfection shows promising results for *in vitro* and *in vivo* non-viral gene delivery and biomedical engineering applications. Data from this study could to be used for MNP drug delivery strategies, ultimately for clinical translation.

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"Always, always you go through the fire, to reach the shine"
Odysseus Elytis, 1979 Nobel Prize in Literature

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Abbreviations

AAV	Adeno-associated viruses
AMAXA	Amaxa® Nucleofector® Technology
bFGF	Basic fibroblast growth factor
BMA	Bone marrow aspirate
CFTR	Cystic fibrosis transmembrane regulator
CFU-Fs	Colony forming unit-fibroblasts
CM	Conditioned media
ddH_2O	Double distilled water
dH_2O	Distilled water
DMEM	Dulbeco's modified eagle medium
DMSO	Dimethyl sulfoxide
DOTAP	dioleoyl trimethylammonium propane
DOTMA	dioleylpropyl trimethylammonium chloride
dsDNA	Double-stranded DNA
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FCS	Foetal calf serum
GFP	Green fluorescent protein
hESC	Human embryonic stem cell
hESCs	Human embryonic stem cells

HGRGT	Human germ line gene therapy
hMSC	Human mesenchymal stem cell
hMSCs	Human Mesenchymal stem cells
hTERT	Human telomerase reverse transcriptase
KO-DMEM	Knock out-Dulbeco's modified eagle medium
KO-SR	Knock out-Serum replacement
LF2000™	Lipofectamine 2000
MEF-CM	Mouse embryonic fibroblast-conditioned media
MEFs	Mouse embryonic fibroblasts
MG-63	Human osteosarcoma fibroblasts, cell line
MNP	Magnetic nanoparticle
MNPs	Magnetic nanoparticles
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cells
mT	Millitesla
MW	Molecular weight
NCI-H292	Human lung mucoepidermoid carcinoma cells, cell line
NdFeB	Neodymium iron boron
NEAA	Non essential amino acids
NIH-3T3	Mouse embryonic fibroblasts, cell line
nm	Nanometers
Oct-4	Octamer-binding protein-4
PBS	Phosphate-buffered saline

pCIKLux	Plasmid pCIKLux expressing luciferase reporter gene
pEGFP-N1	Plasmid EGFP-N1 enhanced expressing green fluorescent protein
PFA	Paraformaldehyde
PSA	Penicillin-Streptomycin-Amphotericin B
RNAi	RNA interference
RISC	RNA-induced silencing complex
RT	Room temperature
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SF	Serum free
SF-DMEM	Serum-free DMEM
SGT	Somatic gene therapy
siRNA	Small interfering RNA
SOX1	Sex determining region Y-box 1
SPION	Superparamagnetic Iron Oxide Nanoparticle
ssDNA	Single-stranded DNA
SSEA-1	Stage-specific embryonic antigen-1
SSEA-4	Stage-specific embryonic antigen-4
ssRNA	Single-stranded RNA
SV40	Simian vacuolating virus 40

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1. **Fouriki A**, Clements MA, Farrow N, Dobson J (2011). Nanomagnetic gene transfection: The efficiency of oscillating magnet arrays and nanoparticles for gene delivery. *Histology and Histopathology: Cellular and Molecular Biology*, 26 (1). TERMIS EU Annual Meeting, Granada, Spain.
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2. **Fouriki A**, Clements MA, Farrow N, Dobson J (2011). Nanomagnetic gene transfection: The efficiency of oscillating magnet arrays and nanoparticles for gene delivery. 4th annual scientific conference UK NSCN, York, UK.
3. **Fouriki A**, Clements MA, Farrow N, Dobson J (2010). Enhanced nanomagnetic transfection of human mesenchymal stem cells using oscillating magnet array techniques. 4th UK Mesenchymal Stem Cell Meeting, Leeds, UK.
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Chapter 1

Literature Review

1.1 Preface and chapter synopsis

The purpose of regenerative medicine is to develop new therapies for the repair or regeneration of human cells, tissues or organs and to restore or enhance normal function (Mason, 2008; Couto *et al.*, 2012). This field is fast expanding and by its definition largely multidisciplinary. One of several well known, high impact advances of regenerative medicine that build our hopes and future expectations include the Nobel Prize winning discovery that mature cells can be reprogrammed back to pluripotent stem cells (Takahashi & Yamanaka, 2006). Furthermore, and reaching the clinic, lifesaving technologies have led to the first successful transplantation of a human tissue engineered trachea (Macchiarini *et al.*, 2008), followed by successful transplantation of the first tissue engineered organ, a trachea (Jungebluth *et al.*, 2011).

Such breakthroughs are multifactorial tasks, and scientists along with clinicians are working closely to face the many challenges and needs in human cell therapies, gene-based methods and molecular biology, engineering, biomaterials and advanced surface chemistry. Gene therapy has been proven a useful tool for the delivery of needed growths factors or biomolecules that traditionally hold an essential part in multiple regenerative medicine applications (Harrison *et al.*, 2014).

This chapter aimsin providing the reader with the essential understanding of the general background of this study. The focal point and this work's primary interest, the nanomagnetic gene transfection method as a magnetic nanoparticle (MNP)-based gene delivery tool, has been introduced and discussed. In addition, and as a requirement in order to understand the series of progressions that led to the development of the method, the key

terms of gene therapy and its significance as a potential breakthrough therapeutic approach, as well as the major currently available viral and non-viral approaches for gene delivery were described. Furthermore, the physical background of magnetism, magnetic materials and magnetic nanoparticles related to nanomagnetic gene transfection has been introduced along with some of their multiple applications. Finally, the human and mouse cell culture systems (primary cells and cell lines) used in this study for the application of the method and evaluation of the qualitative and quantitative results were presented in this chapter.

1.2 Overview of gene therapy

Genes are heritable units controlling identifiable traits in every organism and for a long time have been considered as medicines (Mammen *et al.*, 2007; Wirth *et al.*, 2013). Living beings rely on genes coding for all proteins, following the central dogma of molecular biology to ensure specific transcription from DNA to RNA and translation from RNA to protein (Crick, 1970;). In September 1966, Joshua Lederberg talked about the necessity of evolutionary theory to model a self-modifying system and the potential of genetic therapy to repair any "genetic-metabolic" disease (Lederberg, 1966). Nowadays, gene therapy is considered as a method to insert genetic material into an individual's cells and tissues in order to replace a defective gene, or by introducing a new function into cells to attempt to treat complex diseases at a genetic level (Kay, 2011; Soleimani *et al.*, 2015). The diseases targeted by gene therapy are some of the most complex and deadly such as cancers, inherited or acquired genetic deficiencies, autoimmune diseases, cardiovascular disease and Parkinson's (Ginn *et al.*, 2013). Gene therapy as a potential therapeutic tool requires

effective gene delivery via vectors, such as viruses and plasmids, which are agents used as vesicles to carry foreign genetic material into the target cells and tissues (Giannoukakis *et al*., 1999; Grigsby & Leong, 2010). Gene therapy is usually divided into the two main classes of somatic gene therapy (SGT) and human germ line gene therapy (HGLGT) that are fundamentally different with respect to long-term consequences. HGLGT involves intervention at the embryonic stage in order to tackle inherent genetic disorders, potentially with the transgene (the transferred gene), being passed on to future generations, which has given rise to numerous ethical issues and controversies over time (Nielsen, 1997; Jones, 2006; Soleimani *et el.,* 2015). The next paragraphs will focus on SGT and its significant input in regenerative medicine.

1.2.1 Somatic gene therapy (SGT)

Somatic gene therapy is the only technique that is currently considered appropriate for use in humans. It involves the insertion of genes into somatic cells of an individual with a life-threatening genetic disease that is intended only to eliminate the clinical consequences of the disease. The genetic intervention affects only a subset of patient's cells and passes no genetic information to the next generation. Therefore, it does not directly affect any descendents (Mountain, 2000; Mavilio & Ferrari, 2008).

1.2.1.1 Criteria for SGT candidates

In SGT, gene transfer systems are chosen and modified based on each therapeutic target's particular needs. The multiple criteria which enable a particular disorder to be an appropriate candidate for SGT include the following:

- Severity and frequency of the disease that can have a dramatic impact on patient's quality of life.
- Nature of the genetic defect or abnormality. For example, monogenic diseases are appealing due to the need of replacing well-defined defective genes (Ginn *et al.*, 2013).
- Lack or ineffectiveness of already existing treatments (Mavilio & Ferrari, 2008).
- Existence of a suitable experimental animal model to enable research and develop safe protocols.
- Availability of a suitable gene transfection system for the affected cell type.
- Ability to isolate and clone the defective gene.
- Introduction of the normal gene into a significant subfraction of the affected target tissue, to ensure sufficient gene expression for the desirable effect) (McBain *et al.*, 2008a).

1.2.1.2 The ex vivo approach

In SGT, genes can be delivered by two different approaches, the ***ex vivo*** and the ***in vivo***:

The ***ex vivo*** approach is the most common where all genetic alterations of patients target cells are performed outside the body. Firstly, the physiologically accessible target cells are removed from the patient. These cells are manipulated in the laboratory in order to

incorporate the vector containing the transgene or recombinant vector and then are introduced back into the patient (Figure 1.1) (Mammen *et al.*, 2007). This approach is likely to be more efficient due to the accumulation of a higher vector to target cell ratio present in cell culture, and because it allows the reintroduction only of those cells back into the patient that have incorporated the transgene, improving efficacy (Naldini, 2011). Additionally, viral and non-viral delivery systems that have been extensively developed for gene therapy applications are being used for the vector reintroduction back to the patient (Cox *et al.*, 2015). Furthermore, there is better control over the dosage of therapeutic molecules to be delivered to cells (Hsu *et al.*, 2013).

However, there are two main disadvantages of the *ex vivo* approach. First, invasive procedures are used for the extraction and reintroduction of cells, with a potentially poor engraftment following reintroduction back into the patient that could compromise the effectiveness of the treatment (Tuszynski, 2007; Cox *et al.*, 2015). Second, the use only of cells capable of surviving outside the body, being manipulated in cell culture and transplanted back to target tissues while retaining their properties and function (Cox *et al.*, 2015).

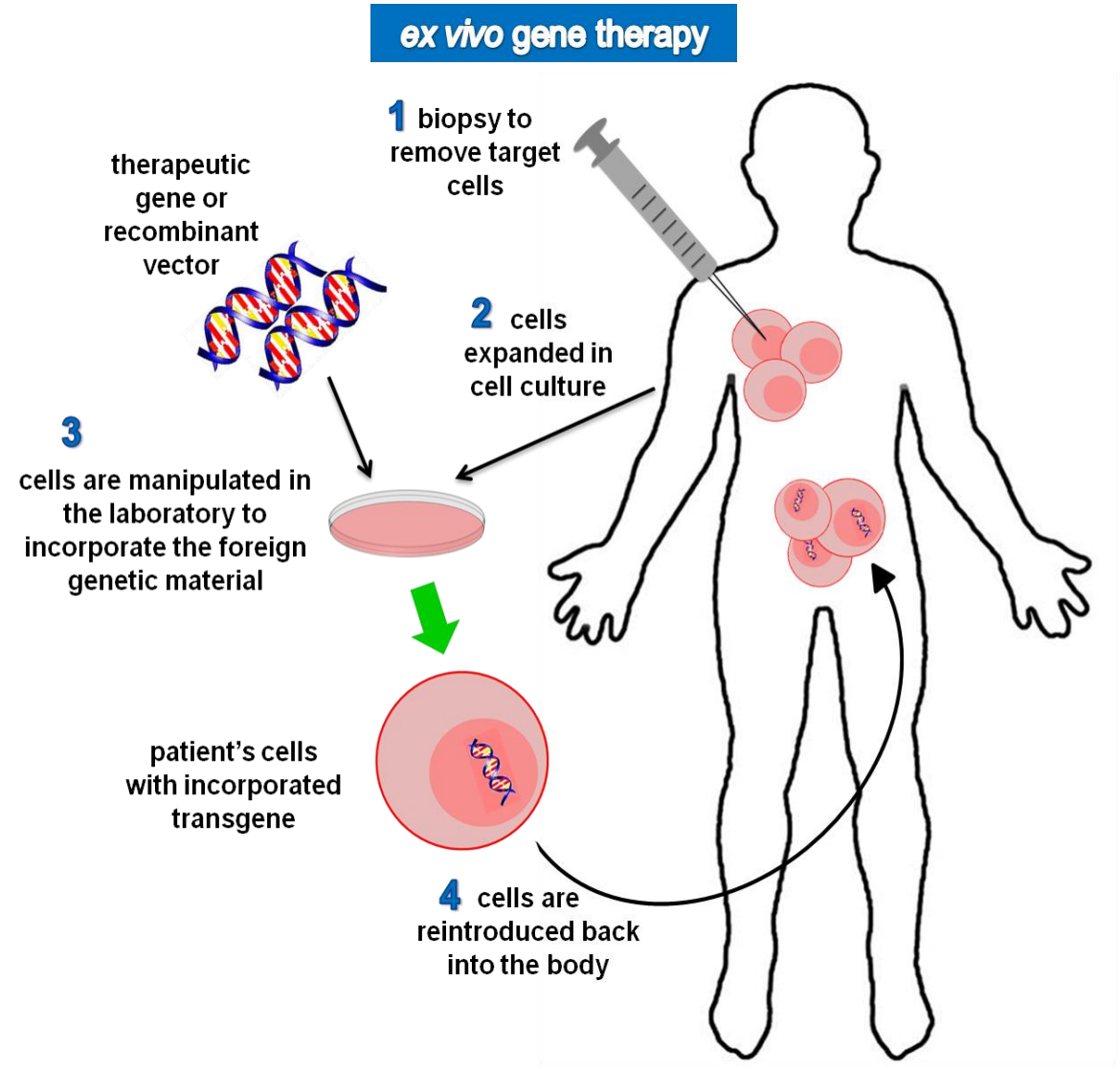


Figure 1.1: Flow chart of key steps during ex vivo somatic gene therapy. (1) Target cells are removed from patient with biopsy and (2-3) cultured and manipulated in the laboratory to incorporate the therapeutic gene or recombinant vector. (4) Cells with incorporated transgene are reintroduced back into the body to produce the desired protein encoded by the inserted gene.

1.2.1.3 The in vivo approach

The ***in vivo*** approach allows direct administration of gene transfer vectors to patients in their native tissues, called *in situ*, that are still part of a living organism (Figure 1.2). This is contributing in the simplicity of the system and repeatability of administration in the same location (Tuszynski, 2007). s. This method is less invasive compared to *ex vivo* and advantageous for working with less accessible cells or cell types which do not easily survive for long outside the body (Duan *et al.,* 2004).

Thetechnique comprises two major limitations. The first lies with immune system response and rejection of therapeutic vector or toxic reactions (Raper *et al.,* 2003; Bessis *et al.,* 2004; Manno *et al.,* 2006; Mavilio & Ferrari, 2008; Naldini, 2011). The second refers to non-specific gene targeting, when this is not the aim, which is a significant challenge since the vector moves around the body until it reaches the target tissue that could lead to a number of consequences such as: (i) the possibility of the vector infecting other tissues or the germ line , (ii) the insufficiently controlled vector distribution and dosage, and (iii) the potential for insertional mutagenesis or off-target mutations (Tuszynski, 2007; Howe *et al.,* 2008; Cox *et al.,* 2015).

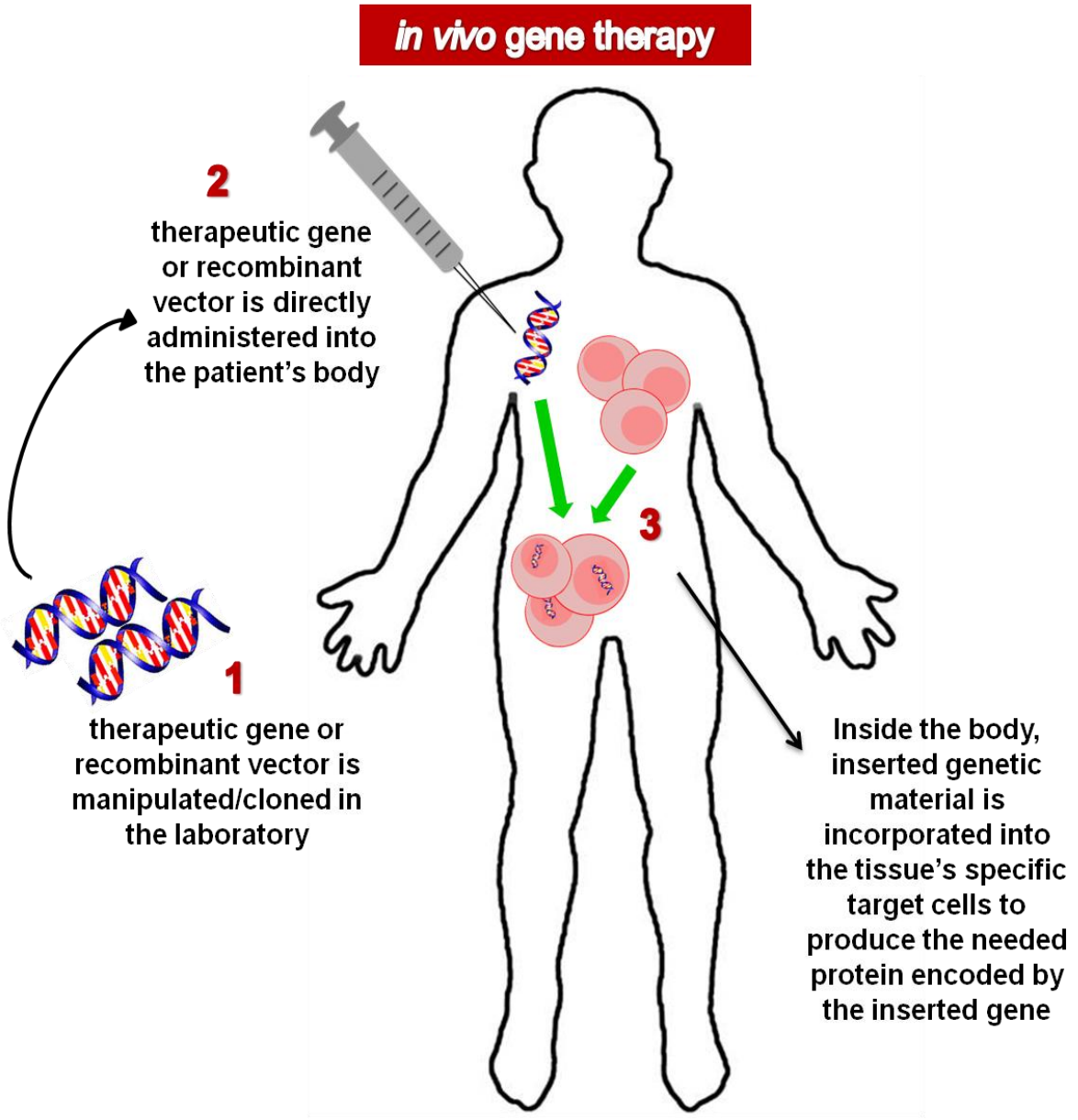


Figure 1.2: Flow chart of key steps during in vivo somatic gene therapy. (1) The therapeutic gene or recombinant vector carrying the gene of interest is manipulated in the laboratory and (2) introduced directly into the patient's body. (3) Inside the body and in contact with the specific tissue's cells the transgene is incorporated into the target cells to produce the desired protein encoded by the inserted gene.

1.2.2 Success stories in clinical trials

Gene therapy by its definition, and due to the ability of acting on a single cell basis, has a wide therapeutic potential that could provide more effective treatment in some existing therapeutic options or alleviate and even cure previously untreatable diseases. The input of gene delivery for gene therapy in the preclinical research is truly extended and diverse. However, there are several gene therapy success stories that have come a long way from basic science in being translated to therapies in the clinic, some of which were presented in this section.

1.2.2.1 Adenosine Deaminase-Severe Combined Immuno-deficiency (ADA-SCID)In 1990, the first gene therapy trial was conducted treating successfully but temporarily a 4-year-old girl diagnosed with Adenosine Deaminase-Severe Combined Immuno-deficiency (ADA-SCID), by *ex vivo* retroviral-mediated introduction of the normal ADA gene into T lymphocytes (Blaese, 1993). Since 2000, more than 30 patients with ADA-SCID have been treated worldwide, with reconstruction of immune function and no observed adverse events related to the gene transfer technology (Ferrua *et al.*, 2010; Ginn *et al.*, 2013).

1.2.2.2 X-linked Severe Combined Immuno-deficiency (SCID)

In 2000, the first successful treatment of 2 infants suffering from X-linked SCID (SCID-X1) was reported (Cavazzana *et al.*, 2000). Since then another 17 infants in two clinical trials have been treated by *ex vivo* retroviral-mediated restoration of the missing IL2RG gene, experiencing full immunological reconstruction that highlighted the therapeutic

alternative for patients with no suitable matching donor (Hacein-Bey-Abina *et al.*, 2002; Gaspar *et al.*, 2004; Ginn *et al.*, 2005; Ginn *et al.*, 2013).

1.2.2.3 Melanoma and Sarcoma

In 2006, objective regression of metastatic melanoma in 2 patients and sustained levels of engineered cells' circulation one year later were reported, following successful immunotherapy with *ex vivo* retroviral transduction of autologous T lymphocytes capable of tumour recognition (Morgan *et al.*, 2006). By extending this strategy, in 2011, a similar clinical trial reported objective clinical responses in 4 patients with synovial cell sarcoma and in 5 patients with melanoma, indicating the effectiveness of T-cell receptor-based gene therapies (Robbins *et al.*, 2011).

1.2.2.4 X-linked Adrenoleukodystrophy (X-ALD)

In 2009, following long term follow-up, significant clinical benefit was reported for 2 boys treated for X-linked adrenoleukodystrophy (X-ALD), a severe brain demyelinating disease caused by ALD protein deficiency encoded by ABCD1 gene. Lentiviral-mediated *ex vivo* gene transduction in haematopoietic cells demonstrated the stoppage of progressive demyelination, which was comparable to the results from allogeneic haematopoietic cell transplantation (HCT) as the only other available treatment (Cartier *et al.*, 2009).

1.2.2.5 Leber's Congenital Amaurosis (LCA)

In 2010, gene therapy for Leber's congenital amaurosis (LCA), an inherited retinal blinding disease, was reported as safe and effective using sub-retinal administration of adeno-associated (AAV) virus encoding the mutated RPE65 gene. The underlying improvement of both objective and subjective vision measures was supported by three independent studies (Simonelli *et al.*, 2010).

1.2.2.6 β-thalassaemia

Also in 2010, in a trial for β-thalassaemia which is characterised by reduced synthesis of haemoglobin, an adult patient following *ex vivo* lentiviral β-globin gene transfer became transfusion independent for 21 months, while since childhood monthly transfusions were required (Cavazzana *et al.*, 2010).

1.2.2.7 Chronic Lymphocytic Leukaemia (CLL)

In 2011, the treatment of 3 patients with chronic lymphocytic leukaemia (CLL) was reported using autologous genetically modified T lymphocytes to express chimeric antigen receptors (CAR) that target tumour-associated cell surface antigens, indicating potent antitumor effects (Kalos *et al.*, 2011).

1.2.2.8 Heart Failure

Also in 2011, in a trial with 39 patients suffering from advanced heart failure, the upregulation of calcium (Ca^{2+}) by administration of AAV-mediated sarcoplasmic

reticulum Ca^{2+}-ATPase, alleviated considerably heart failure symptoms (Jessup *et al.*, 2011; Hammoudi *et al.*, 2015).

1.2.2.9 Haemophilia B

In 2012, gene therapy for Haemophilia B was reported as safe and effective for 2 patients treated with AAV-mediated transduction of the lacking factor IX controlling blood clotting. Additionally, all 7 patients of this trial showed no acute or long-lasting toxicity, and experienced the beneficial effects of factor IX levels elevation eliminating or reducing the need for regular factor IX infusion (Tuddenham, 2012).

1.2.2.10 Lipoprotein Lipase (LPL) deficiency

Also in 2012, Glybera was recommended from the European Medicines Agency (EMA) as the first gene therapy product to receive authorisation and reach the clinic for the treatment of lipoprotein lipase (LPL) deficiency, in patients with severe or multiple pancreatitis attacks (Ylä-Herttuala, 2012).

1.2.3 Hurdles and prospects

Success stories such as the above required decades of efforts and countless hurdles to be overcome. In some cases, disappointments could not be prevented that even affected patients' welfare. Some of these cases described non-responding patients and specifically 15 with metastatic melanoma, 2 with synovial cell carcinoma and 6 with melanoma (Morgan *et al.,* 2006; Robbins *et al.,* 2011), while 4 patients had developed leukaemia but eventually treated in SCID-X1 trials (Hacein-Bey-Abina *et al.,* 2008; Howe *et al.,* 2008). However, the most disheartening were the 3 cases of patient deaths; (i) the first, in 1999 4 days following adenoviral transduction for X-linked ornithine transcarbamylase (OCT) deficiency (Raper *et al.,* 2003); (ii) the second, who developed leukaemia 30 months out of treatment for SCID-X1 following the gene-therapy protocol stoppage in 2002 (Howe *et al.*, 2008; Hacein-Bey-Abina *et al.,* 2010); and (iii) the third, in 2016 7 days following repeated higher doses of a drug aiming to treat anxiety and motor disorders associated with Parkinson's disease as well as chronic pain in people with cancer (Butler & Callaway, 2016).

Nevertheless, and quite rightly, the scientific world has invested resources and hopes in gene therapy, and a long list of treatment schemes for various target diseases have reached the stage of clinical trials. The vast majority of clinical trials has been devoted to cancer (64.4%), followed by monogenic, cardiovascular and infectious diseases addressing approximately another 25% of the trials (Figure 1.3) (Ginn *et al.,* 2013).

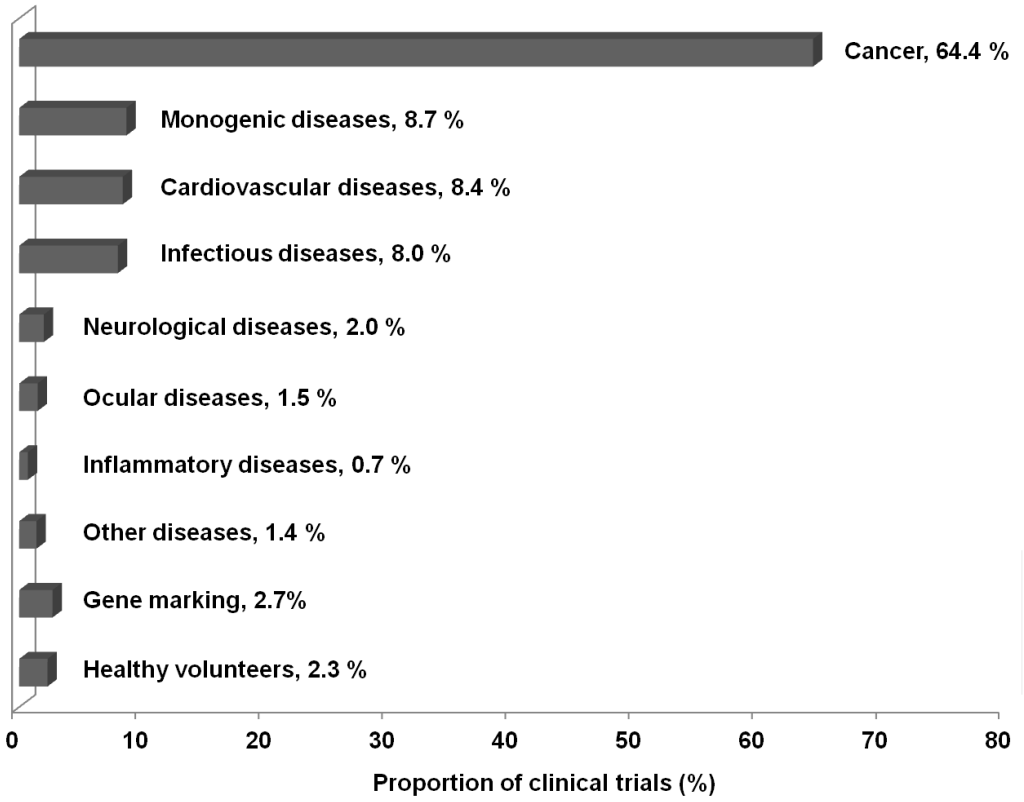


Figure 1.3: Diseases targeted by gene therapy in clinical trials. The figure is indicative of the range and proportion of diseases addressed by gene therapy clinical trials worldwide up to 2012 (Adapted from Ginn et al., 2013).

Furthermore, there are diseases such as Cystic Fibrosis (CF) and Parkinson's disease (PD) where no cure is yet available, but they are considered good candidates for gene therapy and might be possible to be addressed using cell-therapies (Mason & Dunnill, 2008).

CF is caused by a single gene mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) and clinically leads to inflammation and lung disease due to airways blockage by mucus build-up and decreased mucociliary clearance. Related clinical trials have began more than 20 years ago employing viral and non-viral vectors, extending our

understanding and marking the difficulty of gene delivery into the lungs (Griesenbach *et al.,* 2006; Flotte, 2007; Mueller *et al.,* 2008; Mimoto *et al.,* 2010). More recently, it was reported that lentiviruses may be capable of evading the immune system which offers new hope for CF gene therapy prospects (Griesenbach *et al.,* 2011).

PD presents several features with the major motor symptoms being associated with dysfunctions in brain cells' nuclei that can be effectively targeted by viral vectors (Lim *et al.,* 2010). Extended research has been devoted and progress has been made up to date, were multiple clinical trials have used viral vectors to deliver therapeutic transgenes to neurons, as it is believed that not only the correction of motor symptoms, but also the delivery of neuroprotective genes to specific brain cells nuclei could possibly be a way to stop the disease progression (Kaplitt *et al.,* 2007; Goune *et al.,* 2012; Palfi *et al.,* 2014; Soleimani *et al.,* 2015). Despite the progress made so far and the significant clinical successes, a greater understanding of the technical barriers and true benefits of gene therapy is essential to provide optimized clinical treatments that could address more diseases.

1.3 Overview of gene delivery

Until this day, many different viral and non-viral gene delivery methods have been developed, the most popular of which are illustrated in Figure 1.4. These protocols initially have being tested *in vitro* for their gene transfer efficiency and have proved to be an invaluable tool for understanding genetic pathways, diseases and drug screening (Dykxhoorn *et al*., 2003; Ostedgaard, et al., 2005; Nishijima *et al*., 2007). In addition, reporter DNA constructs, such as firefly luciferase and green fluorescent protein (GFP), are often incorporated into the vector sequence to facilitate sensitive monitoring of gene transfer and validate transfection efficiency following their expression into the target cells (Baldwin, 1996; Welsh & Kay, 1997; Nightingale *et al.,* 2006; Fouriki *et al.,* 2014; Wang *et al.,* 2014). Further *in vivo* experiments using many of these gene transfer methods have been applied in animal models (Mikata *et al*., 2002; Shi *et al*., 2006; Horimoto et al., 2006; Callejas *et al.,* 2013) to extend our understanding and ensure safety prior to human testing (Kalka & Baumgartner, 2008; Aiuti *et al*., 2009). The criteria for choosing the most suitable gene delivery vector (Section 1.3.1), as well as the major currently available methods for gene transfer divided into two main classes of viral (Section 1.4) and non-viral gene delivery (Section 1.5) will be described in the following sections.

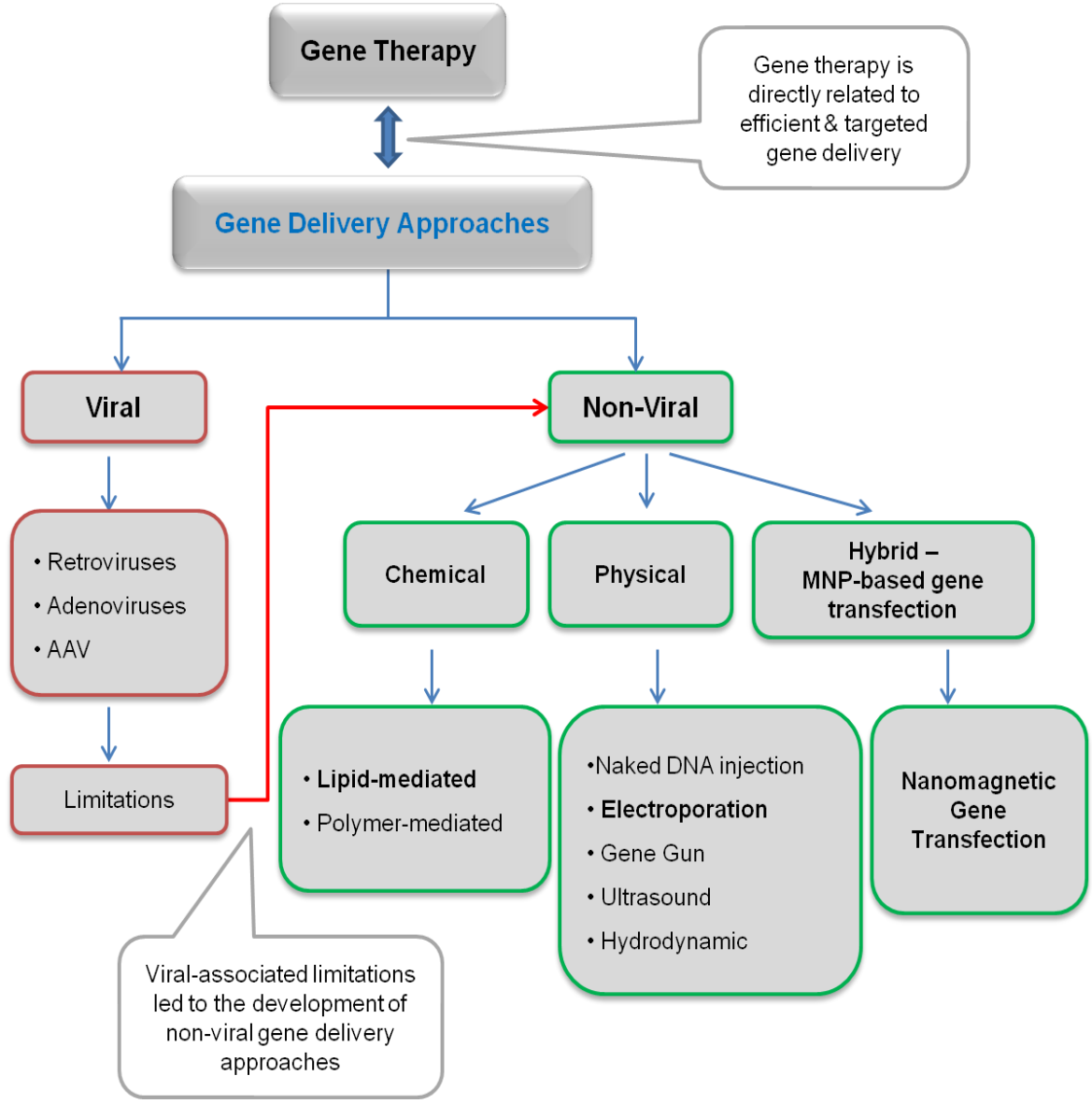


Figure 1.4: Flowchart of major gene delivery approaches. The outline of viral and non-viral gene delivery approaches as a result of the need for gene therapy.

1.3.1 Gene delivery challenges and the choice of vector

The need for further improvement of the currently available gene delivery systems remains, as there are multiple barriers to be overcome described by several research groups that include: (1) the off-target effect, related to the need for increased target tissue

specificity; (2) the host immune response including enzyme degradation of the transgene in serum and reticuloendothelial system (RES) recognition; (3) the insufficient vector accumulation at the target sites that lead to insufficient transgene gene delivery into the cells; (4) the vector uptake by the target cells; (5) the endosomal escape; (6) the nuclear entry and transgene expression (Scherer *et al*., 2002; McBain *et al*., 2008a; Al-Dosari & Gao, 2009; Kay, 2011; Naldini *et al.*, 2011; Zhang *et al.*, 2012; Mingozzi & High 2013).

As expected due to the complexity of the topic, there is no perfect vector or gene delivery system of any kind that can provide a cure that is safe without any limitation or side effect. The choice of the vector is often dictated by the therapeutic need or the experimental objective. Theoretically, an ideal gene therapy vector could combine characteristics of both viral and non-viral systems in order to meet the many gene delivery challenges (Figure 1.5). Several properties of these systems would need to be brought together as the vector has to be efficient, specifically targeted, biodegradable or easy to remove, stable in the blood stream and non-toxic (Munoz *et al.,* 2012). Additionally, it is of high importance to be safe and to initiate no inflammatory or immunogenic response, to be able to protect genetic materials against degradation during transport and be stable for storage (Kay *et al.*, 2009; Grigsby & Leong, 2010; Kay *et al.,* 2011).

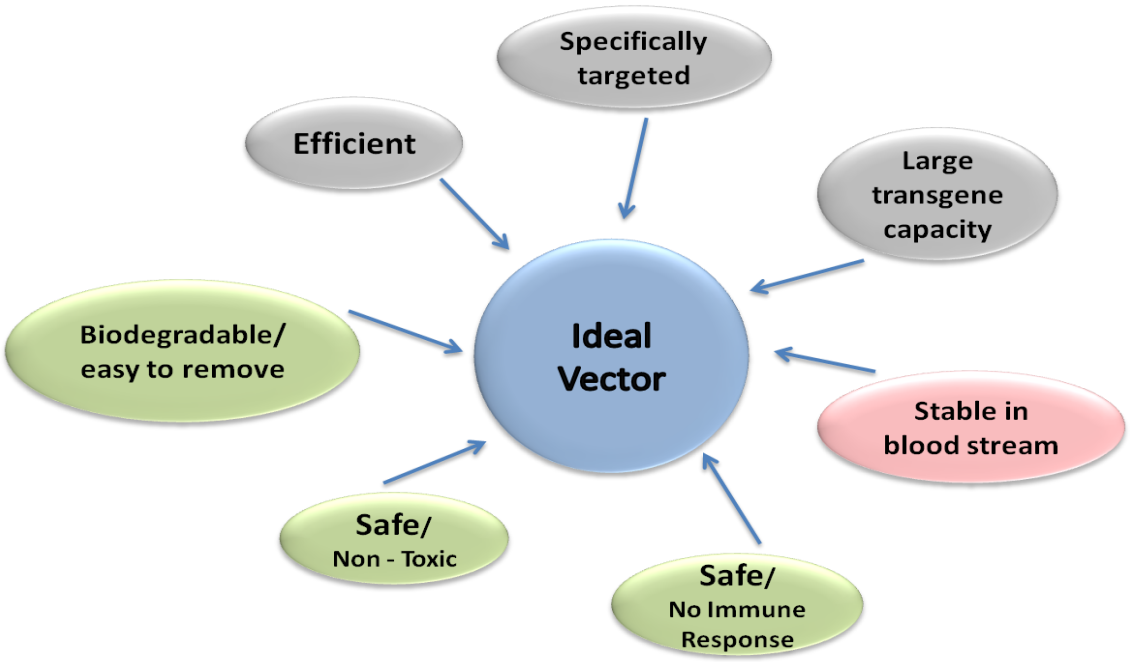


Figure 1.5: Ideal gene transfer vector characteristics for gene therapy. The schematic illustrates the major characteristics desired from a gene delivery vector suitable for *in vitro* and *in vivo* gene therapy.

1.3.1.1 Stable or transient transfection of the vector

Furthermore, the ability of a vector to stably or transiently transfect the target cells is to be considered. For stable transfection the inserted gene is incorporated into the host genome and transgene expression is sustained, affecting cell function and/or regulatory pathways, while for transient transfection the transgene is expressed for only a limited time period (Kim & Eberwine, 2010). The choice for stable or transient transfection is dictated by the short- or long-term experimental objective and/or the therapeutic need. For example, in cases of cell-based gene therapy stable transfection would be needed to ensure that the beneficial modification will not be lost along cell expansion (Wang & Gao, 2014). On the other hand, transient transfection is suitable for short-term applications such as gene

knockdown or silencing with inhibitory RNAs and targeted genomic editing in a sequence-specific way, repairing or disrupting the target region (Mali *et al.,* 2013; Li *et al.,* 2014).

1.4 Viral gene delivery approaches

Viruses (Latin for toxin or poison) are microscopic infectious agents unable to grow or reproduce outside a host cell. Each viral particle, also known as virion, consists of nucleic acid genome (DNA or RNA) that may be single- or double-stranded (Mountain, 2000). They can be considered as packages used to transfer viral genes into host cells (McBain *et al*., 2008a). Once these small parasites infect a cell by receptor-mediated endocytosis, they employ its cellular machinery to maintain their survival in terms of metabolism and nutrient supply and to target the nucleus for the delivery of their genes and regulatory proteins for initiation of transcription and replication (Kay *et al*., 2011; Wang *et al.*, 2014).. Due to such properties, it was a DNA-based polyomavirus SV40 (Simian vacuolating virus 40) that was employed for the first successfully transfected functional gene, thyamidine kinase, into a mammalian cell line (Anderson *et al.*, 1980).

Increasing knowledge about the viral replication mechanism has enabled researchers to modify viruses for distinct scientific uses such as vaccine development (Randrianarison-Jewtoukoff & Perricaudet, 1995; Goedegebuure & Eberlein, 1997). For gene transfer purposes, most viral genes have been replaced within the therapeutic gene cassette to prevent virus replication into the target cell's genome, minimising virulence and to create space for the transgene (Wang *et al.,* 2014). Then, in a process known as transduction, these viral vectors are directed into the target cells and express their functional genetic information (Kay *et al.,* 2011) (Figure 1.6).

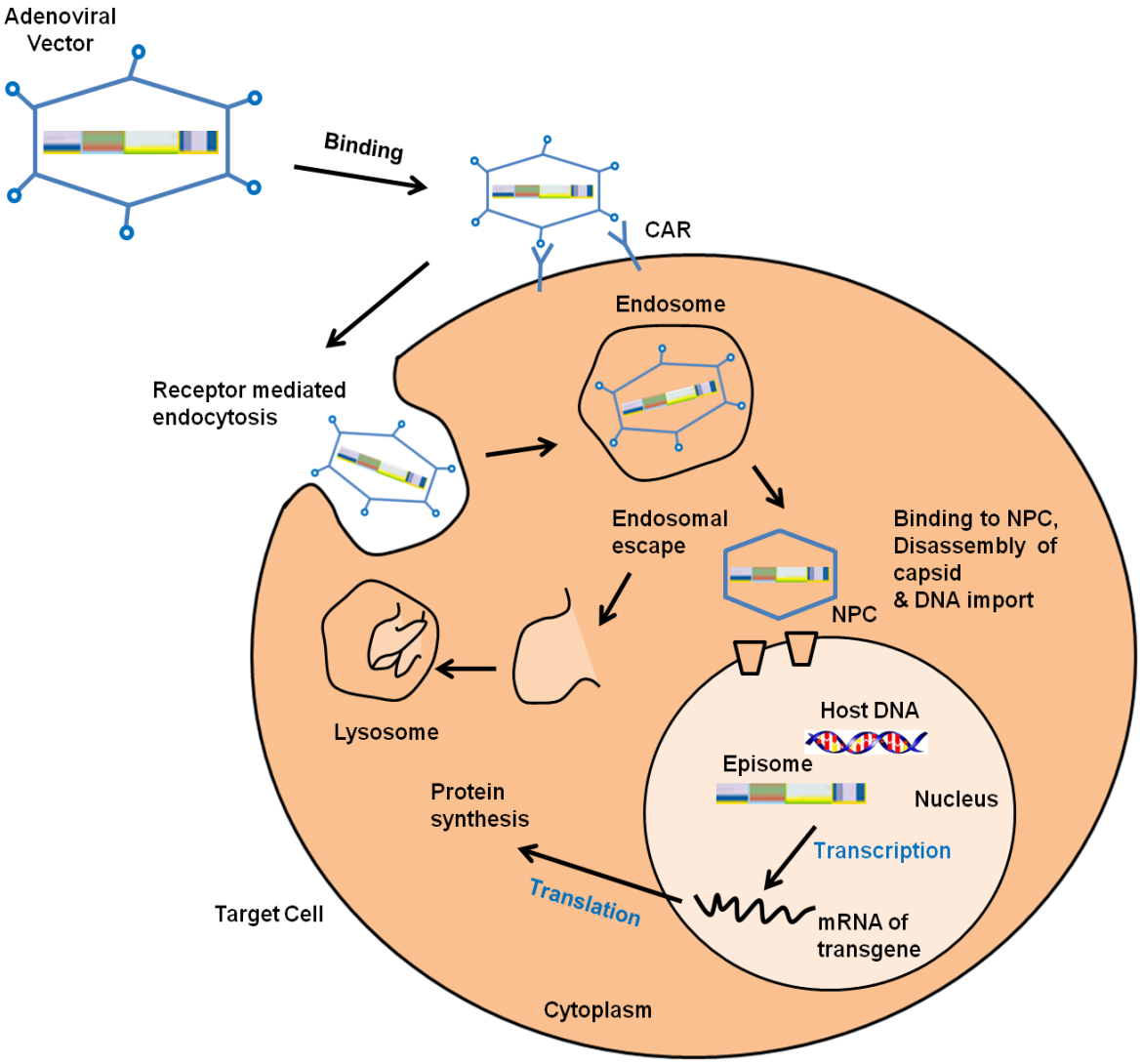


Figure 1.6: Schematic representation of the target cell transduction by an adenoviral vector. The recombinant adenovirus carrying a therapeutic gene binds specifically to the surface of the host cell via a CAR receptor and enters the target cell by receptor-mediated endocytosis. Inside the cytoplasm, endosomal escape and viral capsid disassembly follows. The viral genome is inserted into the nucleus through the NPC and following specific modifications can remain as an episome or be integrated into the host genome. Using the host cell machinery, viral DNA is transcribed and translated completing gene expression.

(CAR; Coxsackievirus and adenovirus receptor, NPC; Nuclear pore complex). (Adapted from Fouriki & Dobson, 2012; Pankajakshan & Agrawal, 2013).

Viral vectors have shown to be important for somatic gene therapy due to their increased overall transduction rate compared to non-viral vectors (Mah *et al*., 2002; Bhattarai *et al*, 2008; Wang *et al.,* 2014). Further molecular engineering of these systems, including protein engineering approaches to generate viral vectors with novel gene delivery capabilities, have been employed trying to meet complicated human therapeutic needs (Schaffer *et al*., 2008). The most commonly used viral vectors for gene delivery as outlined in Figure 1.4, as well as the limitations of their systems are presented in the following paragraphs.

1.4.1 Retroviruses

Retroviruses consist of a linear homodimer of single-stranded RNA (ssRNA) packaged within a lipid-enveloped coat, and normally do not exceed 100nm in diameter. Including the recently developed lentiviruses, retroviruses have high transduction efficiency in many dividing and non-dividing cell types, they can accommodate a transgene size of 7-8kb (and stably integrate the transformed ssRNA to double-stranded DNA (dsDNA) into the host genome (Barton & Medzhitov, 2002; Anson, 2004; Lentz *et al.,* 2012). They have been used successfully in many *in vitro* and *in vivo* studies and clinical trials over the years (Section 1.2.2) (Miller *et al*., 1983; Kay *et al*., 2001; ; Swift *et al*., 2001; Gaspar *et al.*, 2004; Ginn *et al.,* 2005; Aiuti *et al*., 2009; Ferrua *et al.,* 2010). However, a decline in the

use of retroviral vectors in clinical trials from 28% in 2004 down to 19.7% in 2012 has been observed, probably due to the severe adverse events in the SCID trials (Section 1.2.3) (Ginn *et al.*, 2013).

1.4.2 Adenoviruses

Adenoviruses are non-enveloped dsDNA viruses and do not exceed 100nm in diameter. By receptor-mediated endocytosis the virus is inserted into the target cell and reaches the nucleus to initiate transcription and replication (Breyer *et al* ., 2001; Kay *et al*., 2011). Adenoviruses are similar to retroviruses in terms of the wide variety of dividing and non-dividing cell types that are able to transduce as well as their efficiency, and they are considered more advantageous due to their capacity to accommodate larger pieces for foreign DNA (5-36 kb) (Breyer, 2001; Lentz *et al.*, 2011), although their transgene capacity is still limited for certain genes required for clinical applications (Ginn *et al.*, 2013). Adenoviruses have been successfully used in many anti-cancer therapy studies (Breyer, 2001; Molnar-Kimber *et al.*, 1998; Jie *et al*., 2007; Yanagie *et al*., 2009; Ginn *et al.*, 2013), neurodegenerative and cardiovascular diseases and pulmonary hypertension (Breyer, 2001; Horimoto *et al*., 2006). They are the most commonly used vectors in clinical trials covering the 23.3% of all trials (Section 1.2.2) (Ginn *et al.*, 2013).

1.4.3 Adeno-associated viruses (AAV)

Adeno-associated viruses are human parvoviruses that contain single-stranded DNA (ssDNA) packaged into a lipid envelope. They transduce a wide variety of dividing and non-dividing cell types accommodating transgenes 4-5 kb in size and so far they have a

good safety record causing no known human disease (Ostedgaard *et al*., 2005; Kay *et al*., 2009; Qiao *et al*., 2010). They selectively integrate into the host genome and are generally stable (Mulligan, 1993; Carter & Samulski, 2001; Qiao *et al*., 2010). The successes of AAVs *in vitro* and *in vivo* among others include: the microsphere-mediated delivery of recombinant AAV2 for improved vector transduction efficiency (Mah *et al*., 2002; Mah *et al*., 2000), the self-complementary (sf) AAV for intra-articular gene delivery (Kay *et al*., 2009) and the AVV6 use for gene transfer in skeletal muscle (Qiao *et al*., 2010). They have been less widely used in clinical trials (Section 1.2.2) covering the 4.9% of all trials (Simonelli *et al.*, 2010; Tuddenham, 2012; Ginn *et al.*, 2013).

An outline of the characteristics, main advantages and disadvantages of retroviruses, adenoviruses and adeno-associated viruses frequently used for gene delivery is presented in Table 1.1.

Table 1.1: Main characteristics of frequently used viral vectors for gene delivery (Adapted from Lentz *et al.*, 2011 and Soleimani *et al.*, 2015).

Viral Vector	Vector Size (nm)	Genome	Insert Size (kb)	Advantages	Disadvantages
Retrovirus (eg. including lentivirus)	~100	ssRNA	~8	>High transduction efficiency >Prolonged expression >Dividing & non-dividing cell transduction	>Possible pathogenicity at 1st exposure >Insertional mutagenesis
Adenovirus	70-100	dsDNA	~36	>High transduction efficiency >Dividing & non-dividing cell transduction	> Pathogenicity >Transient expression
Adeno-associated virus (AAV)	~20	ssDNA	~4.5	>Prolonged expression >Low immunogenicity >Dividing & non-dividing cell transduction	>Low transduction efficiency

ssRNA = single stranded RNA, dsDNA = double stranded DNA, ssDNA = single stranded DNA.

1.4.4 Limitations of viral vectors

Despite the fact that viral vectors have provided invaluable results for gene delivery and gene therapy, and they have increased significantly our understanding about the field, some general unresolved problems associated with their use include:

(i) safety issues (excluding the AAVs) (Verma, 2000; Hacein-Bey-Albina *et al.*, 2003; Klink *et al*., 2004; Hacein-Bey-Albina *et al.*, 2010; Cox *et al.*, 2015).

(ii) the host inflammatory or immunogenic response that viruses initiate from the infusion of foreign materials (Lu, 2001; Spack & Sorgi, 2001; Raper et al., 2003; Davies, 2006; Pickard & Chari, 2010; Mingozzi & High, 2013).

(iii) the target tissue specificity which can cause tissue damage in undesired tissues and organs (when delivery and transduction is non-specific) (Verma, 2000; Naldini *et al.*, 2011).

(iv) recombination incidents that can lead to the production of a replicating virus causing a direct threat (Hacein-Bey-Albina *et al.*, 2003; Klink *et al*., 2004; McBain *et al*., 2008a).

(v) the limited capacity to accommodate large transgenes and manufacture sufficient stable vector stocks (Breyer *et al*., 2001; Qiao *et al*., 2010; Pickard & Chari, 2010; Kotterman & Schaffer, 2014).

(vi) the inefficiency in transferring the engineered gene into suspension cell lines (Bhattarai *et al*., 2008).

1.5 Non-viral gene delivery approaches

Despite the extensive research and successes in the use of the most popular viral vectors for gene delivery, several limitations and risks remain as discussed (Section 1.4.4), causing the need for further development of non-viral alternative methods.

In general, there are many reasons to explain why non-viral carriers are advantageous and promising some of which are: (i) the simplicity of use and potential for large-scale production, (ii) the low cost of manufacturing and (iii) the large capacity of transgene uptake (Wang & Gao, 2014). Also to their advantage are (iv) their safety in comparison to viral vectors since they contain no disease-causative viral genes, eliminating any chance of viral replication) and (v) the high cell viability following gene transfer depending on the specific non-viral technique used (Martin *et al*., 2005; Guo & Huang, 2012).

Although several hurdles specific to each approach have to be overcome, the main limitation preventing non-viral gene delivery approaches from being more widely used is their considerably lower transfection efficiency compared to the equivalent for viral vectors. Recent advances in the field improving transfection efficiency are shifting the focus towards the potential of non-viral gene delivery approaches that can be divided into three main categories as outlined in Figure 1.4:

- Delivery of genetic materials by the use of **physical methods,** presented in Section 1.3.3.1 (Villemejane & Mir, 2009; Wang *et al.,* 2013).
- Delivery of encapsulated genes mediated by **chemical approaches,** presented in Section 1.3.3.2 (Midoux et al., 2009; Lian *et al.,* 2014).
- Delivery of the gene of interest by the use of multifunctional **hybrid systems** that combine physical and chemical characteristics, such **as magnetic micro- and**

nanoparticle-based gene delivery, presented in Sections 1.3.3.3 and 1.5 (Pankhurst et al., 2003; Dobson, 2006a; Corchero & Villaverde, 2009; Fouriki *et al.*, 2010; Pickard & Chari, 2010; Guo & Huang, 2012; Zhang *et al.*, 2012; Subramanian *et al.*, 2013; Fouriki & Dobson, 2014).

1.5.1 Physical approaches

There are several physical approaches that are being used in the field of non-viral gene delivery. The two most popular approaches of direct injection of DNA and electroporation are described in the next paragraphs. Additionally, other less frequently used physical approaches such as gene gun, ultrasound and hydrodynamic gene delivery are briefly outlined.

1.5.1.1 Direct injection of naked DNA

This method is the simplest non-viral gene delivery system currently in use for gene therapy purposes. It was used for the first non-viral gene therapy clinical trial in 1992 to successfully enhance the immune response against tumours *in vivo* (Nabel, 1992), and remains the most popular non-viral gene delivery system in clinical trials covering the 18,3% of all trials with an increasing trend since 2004 (Ginn *et al.*, 2013). It involves the injection of naked DNA directly into target tissues (eg. skeletal muscle, thyroid, liver, heart muscle, skin and tumour) or into the systemic circulation via a needle or jet that generate pores on the target cell membrane allowing intracellular gene transfer (Niidome & Huang, 2002; Schmidt-Wolf & Schmidt-Wolf, 2003; Gao *et al*., 2007). The benefit of jet injection

relies on the high-pressure stream of liquid that penetrates the skin and targets the tissue of interest. As shown recently, this minimizes patients' discomfort and increases gene expression (Andre *et al*., 2006 ; Villemejane & Mir, 2009). The advantages of naked DNA injection comprise the absence of a biological vector and immunogenicity (Al-Dosari & Gao, 2009). Finally, the limitations include; (i) the low transfection efficiency, (ii) the possibility of some tissue damage or increase of the pressure on the surface of the target cell following injection (Felgner *et al*., 1987; Villemejane & Mir, 2009) and finally, (iii) during systemic circulation the rapid enzymatic degradation due to the action of nucleases and clearance by the uptake of phagocyte system (Schmidt-Wolf & Schmidt-Wolf, 2003; Pankajakshan & Agrawal, 2013).

1.5.1.2 Electroporation

Electroporation (or electropermeabilization) is a versatile, well-studied method by which genetic materials and other molecules can be introduced into cells by a pulsed electric field (Figure 1.7) (Schmidt-Wolf & Schmidt-Wolf, 2003). The short and intense electric shock of electrocompressive forces makes the cell membrane temporarily permeable allowing the DNA to enter the cell (Niidome & Huang, 2002; Villemejane & Mir, 2009;). Electroporation has achieved high transfection efficiency in many tissues and shown good reproducibility *in vivo* compared to other non-viral vectors (Andre & Mir, 2004; Marti *et al.*, 2004). However, the method is limited by a restricted range of electrodes and the lack of accessibility of the electrodes to the internal organs *in vivo* (Al-Dosari & Gao, 2009). In addition, the use of high intensity electric fields can cause permanent changes in the cell membrane structure, resulting in substantial apoptosis or necrosis (Gao *et al*., 2007;

Villemejane & Mir, 2009;). Despite the progress made so far, more research and technological advancements are needed for a wider use in clinical applications.

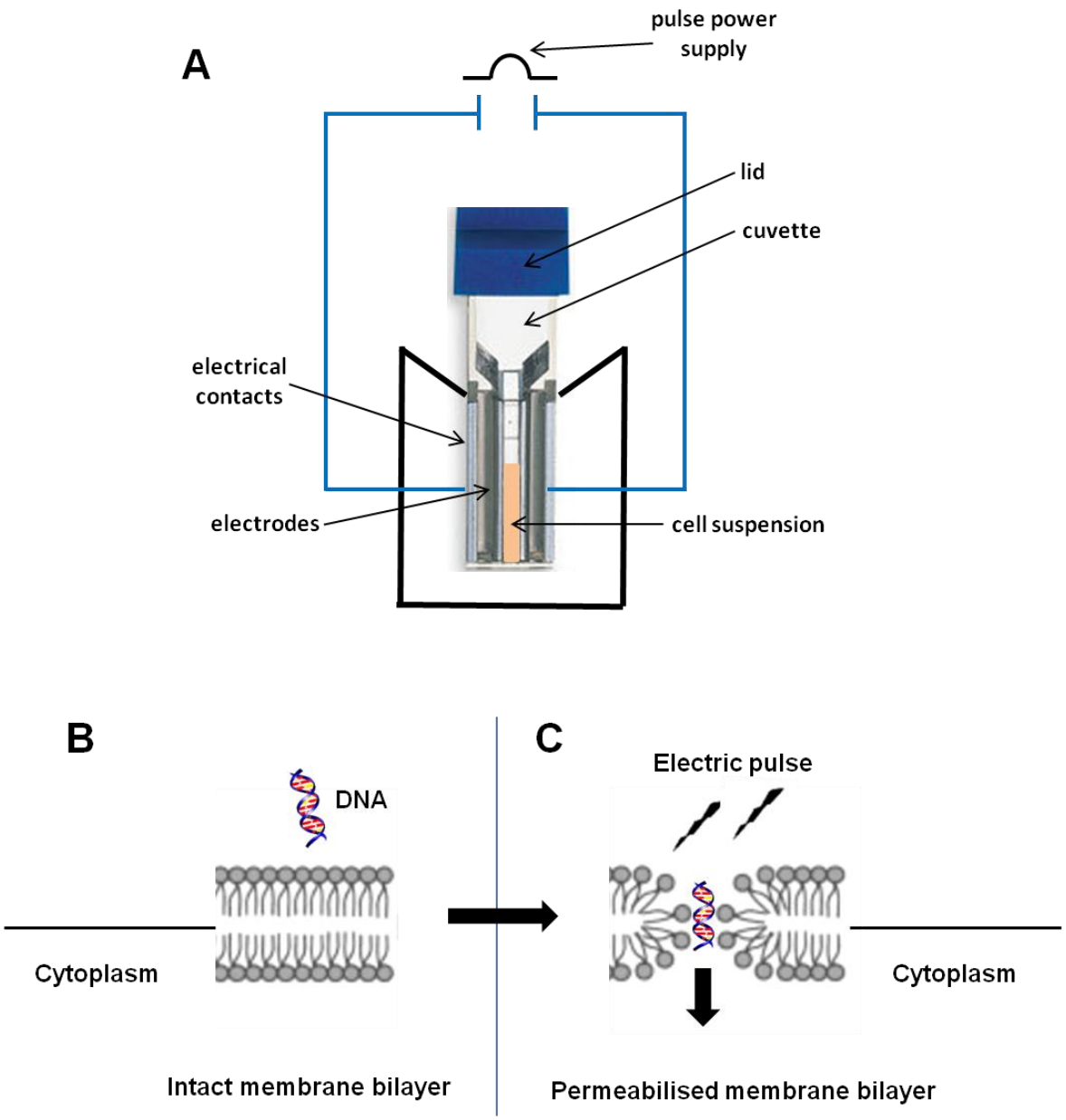


Figure 1.7: Schematic representation of the electroporation process.

(A) A typical cuvette loaded electroporator schematic, where cell suspension is pipetted into the cuvette which has attached one aluminium electrode in each side. (B) Before

electroporation, the target cell membrane lipid bilayer is intact, and (C) the externally applied electrical field causes an increase in electrical conductivity and permeability of the plasma membrane allowing the plasmid DNA to enter the cell.

1.5.1.3 Other methods

Finally, this paragraph briefly summarizes some physical approaches that are less frequently used but research is devoted for their further improvement:

(a) G**ene gun** transfection, involves the targeted tissue being bombarded with heavy metal particles (eg. gold particles) with plasmid DNA deposited on their surface. It has been used in animal studies and a few clinical trials (Al-Dosari & Gao, 2009), with the main limitation being the low penetration of the metal particles. Subsequently, low efficiency in reaching the entire tissue is observed leading to the requirement for surgery to access non-superficial tissues (Villemejane & Mir, 2009).

(b) **Ultrasound**, also known as sonoporation, is a non-invasive suitable for clinical research method that enables gene transfection by generating irradiating ultrasonic waves that temporarily increase cell membrane permeability and allow the internalization of large macromolecules such as plasmid DNA (Niidome & Huang, 2002; Gao *et al*., 2007; Al-Dosari & Gao, 2009). However, it has been reported that it can induce cellular morphology changes, mechanical shear and breakdown of the cytoskeleton causing alterations in cellular mechanisms such as DNA trafficking (Skorpikova *et al*., 2001; Schlicher *et al.*, 2010).

(c) **Hydrodynamic gene delivery,** allows naked DNA to be introduced into the cells in highly perfused internal organs such as the liver and it has been applied only *in vivo* in

animal models with encouraging results (Villemejane & Mir, 2009). The limitation of the method is the high volume of DNA solution required for injection often beyond an acceptable level for patients, and the frequently lethal effects of rapid injection (Gao *et al*., 2007).

1.5.2 Chemical approaches

1.5.2.1 Lipid-mediated gene delivery

Lipid-mediated gene transfer, also known as lipofection, was first developed in 1987 (Felgner *et al*., 1987). It has been successful with *in vitro* and *in vivo* gene therapy experimental models (Schmidt-Wolf & Schmidt-Wolf, 2003; Mintzer & Simanek, 2009), and is the most commonly used non-viral chemical approach. The method has been tested in clinical trials for the treatment of cancer and cystic fibrosis (Schmidt-Wolf & Schmidt-Wolf, 2003; Al-Dosari & Gao, 2009), and currently is the second most frequently used non-viral gene delivery system covering the 5.9% of all clinical trials, after direct nucleic acid injection (Section 1.5.1.1) (Ginn *et al.*, 2013).

Lipid-mediated gene delivery (Figure 1.8) occurs via endocytosis (Mintzer & Simanek, 2009) and involves the assembly of cationic lipids that form liposomes, which are spherical vesicles with one (unilamellar) or more (multilamellar) lipid bilayers surrounding an aqueous core. Its principle relies on the ability of liposomes to encapsulate nucleic acids forming complexes known as lipoplexes that fuse with the cell membrane of the target cell, followed by endosomal escape, release of the gene of interest and its distribution in the appropriate cellular compartment (Guo & Huang, 2012). In case of DNA therapy, DNA has to enter the nucleus for transcription, while for RNA interference (RNAi), small interfering RNA (siRNA) has to remain in the cytoplasm and reach the RNA-induced

silencing complex (RISC) (Huang *et al.*, 2012). Following on from previously mentioned benefits that several non-viral vectors have in common (Section 1.5), this method can protect DNA against enzymatic degradation (Guo & Huang, 2012), as it is released from the intracellular vesicles before being broken down by lysosomal compartments (Gao *et al*., 2007).

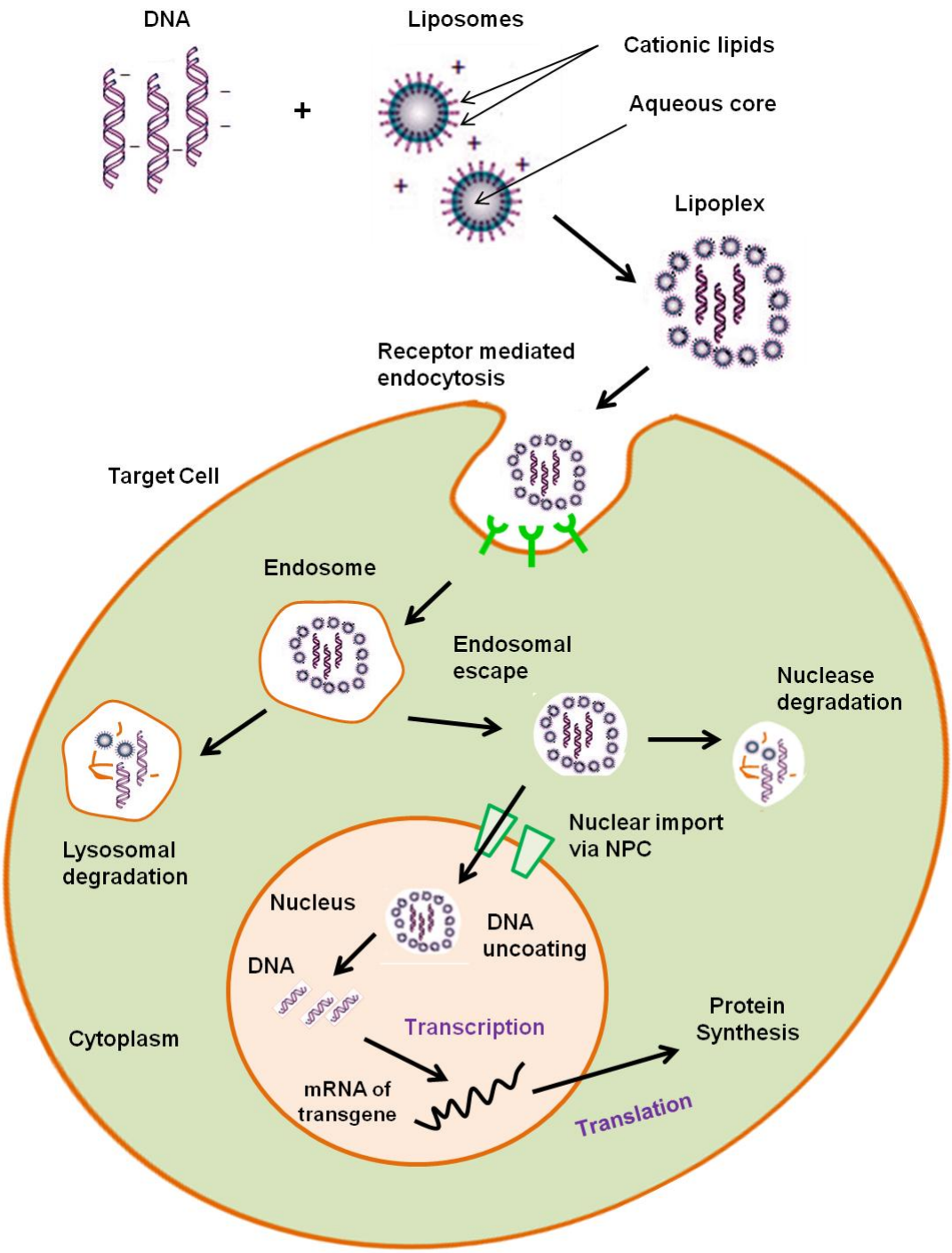


Figure 1.8: Schematic representation of lipid-mediated DNA delivery principle. Cationic lipids that form liposomes, mix and associate with DNA to form spherical lipoplexes, internalized by receptor-mediated endocytosis. Inside the cytoplasm, amounts

of lipoplexes can be degraded by the lysosomes or nucleases, and following endosomal escape the DNA is released and transported into the nucleus through NPC. Inside the nucleus the gene of interest is transcribed followed by translation to complete gene expression. (NPC; Nuclear pore complex). (Adapted from Fouriki & Dobson, 2012; Pankajakshan & Agrawal, 2013).

Cationic-lipid transfection has many advantages but there are drawbacks that require further improvement such as: (i) further increase of transfection efficiency (a common drawback among non-viral systems), (ii) the transient expression that requires repeated doses (when stable transfection is required), (iii) the extended required time for treatment and incubation of cells (4hr to 6hr), (iv) the toxic side effects reported with high doses of lipoplexes administration, (v) the administration route-dependent transfection efficiency *in vivo,* requiring individualized optimization of the vector for distinct target cells, (vi) the potentially high clearance rate from blood stream when positively charged lipoplexes interact non-specifically with serum proteins resulting in their volume increase, (vii) the uptake by the reticuloendothelial system (RES) (Templeton, 2004; Martin *et al*., 2005; Lian *et al.*, 2014).

Multiple studies have attempted to overcome these barriers covered in several review articles (Morille *et al.*, 2008; Guo & Huang, 2012; Zhang *et al.,* 2012). Many cationic headgroup manipulations have been tested and different cationic lipids have been used with two of the most popular being dioleylpropyl trimethylammonium chloride (DOTMA) and dioleoyl trimethylammonium propane (DOTAP) (Al-Dosari & Gao, 2009; Guo &

Huang, 2012). However, modification of cationic lipid molecules has been relatively saturated retaining the need for improved synthetics vectors.

1.5.2.2 Polymer-mediated gene delivery and Polyethylenimine (PEI)

Another actively studied class of synthetic vectors are the cationic polymers that have positively charged groups in their backbone and can interact with negatively charged genetic materials (Fishbein *et al.*, 2010). These polymers mix with DNA or siRNA to form nanosized complexes known as polyplexes, which then enter the target cell by endocytosis employing similar mechanisms described for lipid-mediated gene delivery (Section 1.5.2.1). Generally, polyplexes have some advantages in comparison to lipoplexes that include their small size, narrow distribution and better stability and efficiency in condensing DNA s (Al-Dosari & Gao, 2009; Midoux *et al*., 2009; Dijaj *et al.,* 2014).

Many types of polymers have been developed with the cationic polymer poly-L-lysine (PLL) being one of the first to be used for gene transfer *in vivo* (Gao *et al*., 2007). Additionally, natural biopolymers such as the polysaccharides chitosan and dextran exhibit unique properties such as biodegradability and biocompatibility that increase attention in their gene delivery potential (Raftery *et al.,* 2013).

Among cationic polymers, the most widely investigated **polyethylenimine** (**PEI**), is considered one of the most effective polymer-based transfection agents and its ability to promote gene transfection *in vitro* and *in vivo* was first demonstrated in 1995 (Boussif et al., 1995). PEI exists in linear or branched structures, can undergo functionalized group modifications and can transfect a broad range of cell types (Thomas *et al.,* 2007; Morille *et*

al., 2008; Dijaj *et al.*, 2014). It is advantageous due to its strong DNA condensation capacity, intrinsic endosomal activity and endosomal escape, due to a unique buffering property known as the 'proton sponge effect' that facilitates the release of the transgene into the cytoplasm through osmotic swelling and burst of endosome (Lian *et al.*, 2014; Yin *et al.*, 2014).

Most of the studies have proposed that cationic polymers with high buffering capacities between 4.5 to 7.0 such as PEI, can mediate endosomal escape and ultimately avoid degradation in lysosomes, by transporting protons and chloride ions into the endosome with subsequent osmotic pressure increase and vesicle rupture. The proton sponge mechanism was first proposed in 1997 (Behr, 1997) and despite being debated it remains the most generally accepted mechanism of endosomal escape (Figure 1.9) (Putnam *et al.*, 2001; Akinc *et al.*, 2005; Midoux *et al*., 2009; Benjaminsen *et al*., 2012; Richard *et al*., 2013). Following endosomal escape the plasmid DNA/siRNA is released and distributed in the appropriate subcellular compartment. In the case of DNA therapy, translocation of the DNA into the nucleus for transcription is needed, while for RNAi, siRNA must be delivered to the RNA-induced silencing complex (RISC) in the cytoplasm (You & Auguste, 2010; Guo & Huang, 2012).

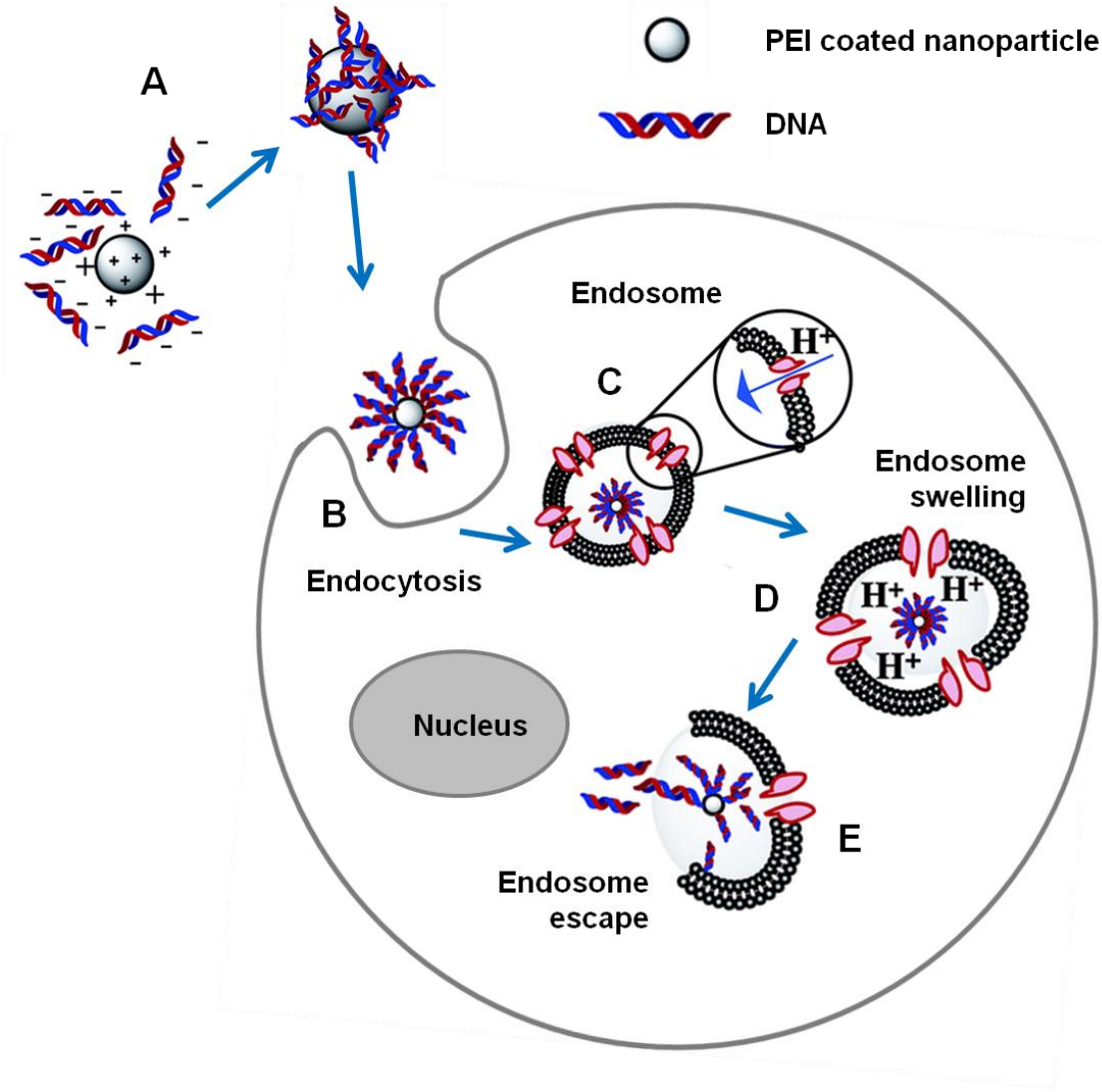


Figure 1.9: Schematic representation of the proton sponge effect of PEI polymer. (A-B) PEI coated nanoparticles mix and associate with DNA to form polyplexes which enter the cell via endocytosis. (C) Inside the cytoplasm, the buffering capacity of PEI induces the pumping of protons into the endosomes, (D) that increases osmotic pressure and endosome swelling, (E) rupturing the endosome and facilitating endosomal escape. The DNA is released into the cytoplasm and transported into the nucleus through NPC (Adapted from Restani *et al.*, 2014).

It has been observed with PEI depending on the choice of its isoform (linear or branched) and molecular weight (MW) complexed with DNA, that different levels of transfection efficiency and toxicity can be obtained both *in vitro* and *in vivo* (Al-Dosari & Gao, 2009). Therefore, PEI transfection efficiency and toxicity are MW- and structure-dependent, with reports showing that high MW PEI can be toxic, while polyplexes made of linear PEI appear more efficient in terms of transfection efficiency and significantly less toxic (Gosselin *et al.,* 2001; Wightman *et al.,* 2001; Zhang *et al.,* 2012). Additional strategies for the improvement of polyplexes can be achieved by conjugation with an inert polymer, for example polyethylene glycol (PEG), or dextran to reduce specific interactions and overall cytotoxicity of PEI-based polyplexes, although transfection efficiency can be compromised (Al-Dosari & Gao, 2009; Tsai *et al.,* 2011). In case of PEG polymer use that can protect particles from agglomeration and macrophage uptake, PEG coating can inhibit fusion with the cell or endosomal membrane reducing the potential for particle cellular uptake and endosomal escape, decreasing transfection efficiency in case of DNA or silencing effect in case of siRNA (Zhang *et al.*, 2012), although several strategies have been employed trying to handle this limitation (Walker *et al.*,2005; Hatakeyama *et al.,* 2007; Hatakeyama *et al.,* 2011).

1.5.3 Hybrid Systems: Magnetic micro- and nanoparticle-based gene delivery

As it was shown in section 1.5, a range of non-viral systems has become available for *in vitro* and *in vivo* gene delivery purposes that due to their physical or chemical characteristics, as well as their acquired technical advances, can eliminate some viral drawbacks, offer significant results and clinical applications. However, their system-specific limitations addressed previously, the lower transfection efficiency compared to some viral vectors, and the challenge of specific targeting to the sites of interest in the body, retain the need for further development of improved carriers for gene delivery for *in vitro* investigations and *in vivo* therapeutic applications.

As illustrated in Figure 1.4 (Section 1.3), a third class of non-viral gene delivery approaches is the emerging field of hybrid systems that includes the magnetic micro- and nanoparticle-based gene delivery combining both physical and chemical characteristics (Pankhurst *et al*., 2003; Dobson, 2006b; Corchero & Villaverde, 2009). This approach involves the use of inorganic (iron oxide) magnetic nanoparticles (MNPs) which are extensively used in biomedicine, easily prepared and surface-functionalized in many ways (Sokolova & Epple, 2008; Prijic & Sersa, 2011; Lian *et al.*, 2014), coated with biocompatible polymers (McBain *et al.*, 2008a; Dijaj *et al.,* 2014), and the presence of externally applied magnetic fields directing gene delivery (Scherer *et al.,* 2002; Plank *et al.*, 2003; Dobson, 2006a; McBain *et al.*, 2008b; Peng *et al.*, 2008; Pickard & Chari, 2010; Fouriki *et al.,* 2012).

In the following sections the background of magnetism and MNPs (Section 1.6) related to magnetic gene/drug delivery and targeting (Section 1.7) will be described, as the basis for

magnetic nanoparticle (MNP)-based gene delivery approach and specifically the nanomagnetic gene transfection system (Section 1.8).

1.6 Magnetics in biomedicine – the foundation for MNP-based gene delivery

1.6.1 Magnetism and magnetic materials

A magnetic field is produced due to the motion of charged particles. Therefore, a field is produced each time an electrical charge is in motion (Jiles, 2015). Magnetic fields based on the SI system are usually measured in units of Tesla (T) and Gauss (G). The phenomenon of magnetism involves the interaction of two magnetic fields and arises from the motion of electrical charges and the interaction between electrons with atoms. Therefore, all materials can be affected by magnetic fields in different ways, and the type of magnetism of each material can be different based on the electronic configuration (Dobson *et al*., 2007). As all materials interact in some way with externally applied magnetic fields, they are categorised based on their magnetic susceptibility as: (a) diamagnetic, (b) paramagnetic, (c) ferromagnetic, (d) antiferromagnetic and (e) ferrimagnetic.

- Diamagnetic materials have all their electron spins paired and a very weak negative magnetic susceptibility. They are considered a very weak type of magnetism displayed by all materials (Dobson *et al*., 2007; Faraji *et al.*, 2010).
- Paramagnetic materials have some uncompensated spins in the absence of a magnetic field and are magnetized only in the presence of a magnetic field were the spin moments are aligned. Similarly, when removed from the applied magnetic field paramagnets have zero net magnetization. The strength of the magnetic field is

proportional to the degree of alignment (Figure 1.10) (Barnes *et al.*, 2007; Dobson et al., 2007).

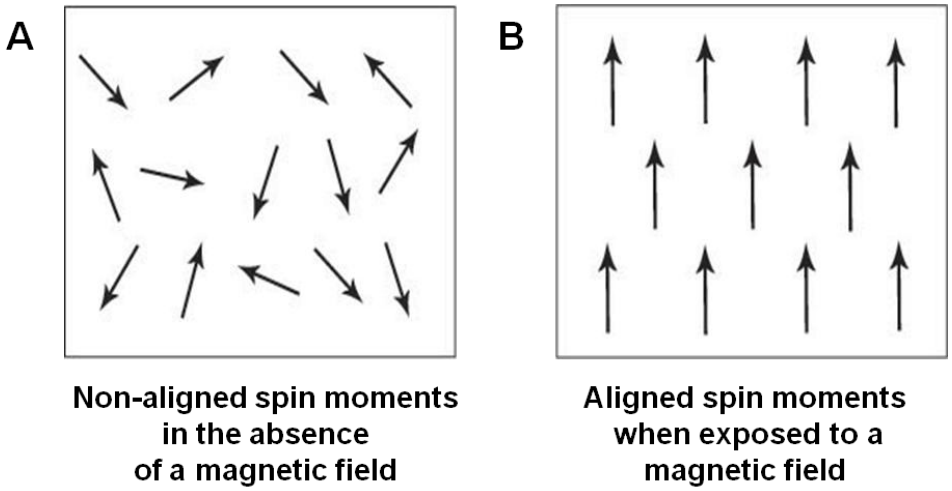


Figure 1.10: Schematic representation of spin moments of paramagnetic materials. (A) Paramagnets have non-aligned spin moments in the absence of a magnetic field and (B) are magnetised in the presence of a magnetic field were their spin moments are aligned (Adapted from Dobson et al., 2007).

- On the other hand, ferromagnetic materials have a strong, positive magnetic susceptibility and aligned spin moments. Unlike paramagnets, ferromagnets remain magnetised even after the applied field is removed (Figure 1.11A) (Barnes *et al.*, 2007; Hofmann-Amtenbrink, *et al.*, 2010).
- Antiferromagnetic materials are special cases of ferromagnetic substances with uncompensated spin moments of equal magnitude coupled antiparallel to one another exhibiting no net magnetization (Figure 1.11B) (Dobson *et al.*, 2007; Hofmann-Amtenbrink, *et al.*, 2010).

- Ferrimagnetic materials like antiferromagnets, have uncompensated spin moments antiparallel to one another, but with unequal magnitude giving rise to strong net magnetization which is also retained after the magnetic field has been removed (Figure 1.11C). An example of ferrimagnet is magnetite (Fe_3O_4) (Dobson *et al*., 2007; Hofmann-Amtenbrink, *et al.*, 2010).

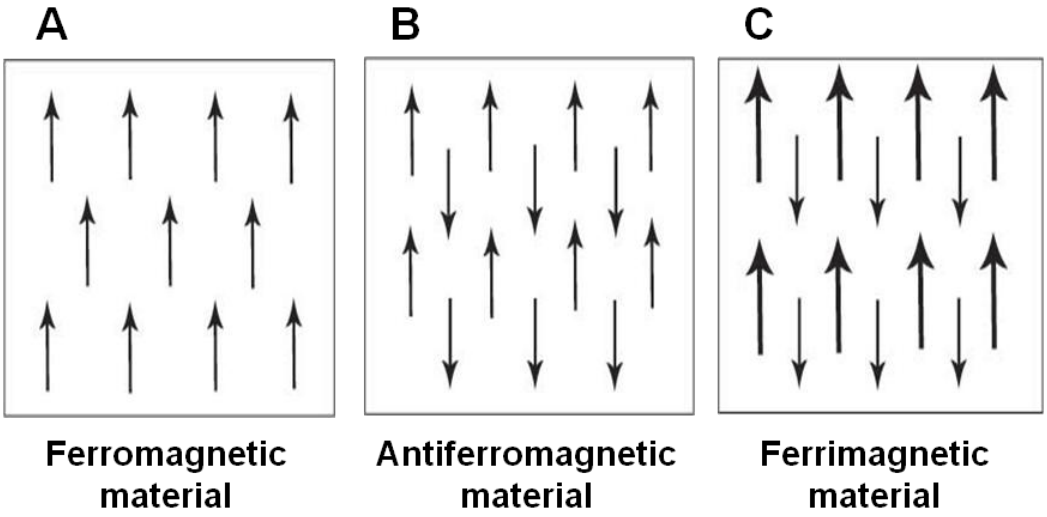


Figure 1.11: Schematic representation of spin moments of ferromagnetic, antiferromagnetic and ferrimagnetic materials. (A) Ferromagnets are permanently magnetised with aligned spins moments. (B) Antiferromagnets have antiparallel spin moments of equal magnitude and no net magnetization. (C) Ferrimagnets are permanently magnetised with antiparallel spin moments of unequal magnitude (Adapted from Dobson et al., 2007).

- Superparamagnetic materials are an unusual case of magnetism. Their particles are very small, in the order of approximately 20 nm in diameter. They have uncompensated spins but the orientation of their individual spin moments can be

ferromagnetic, ferrimagnetic or antiferromagnetic. Their spins are coupled but they can randomly flip direction caused by thermal energy fluctuations. Superparamagnets behave as paramagnets, therefore they retain no magnetization when the magnetic field is removed (Dobson *et al*., 2007; Jiles, 2015). This characteristic is important and superparamagnetic properties are desirable for biomedical applications as the MNPs will not be attracted to each other reducing the risk of agglomeration for *in vivo* therapeutic purposes (Dobson, 2006; Getzlaff, *et al.*, 2008; Mahmoudi *et al.*, 2011).

1.6.2 Magnetic nanoparticles (MNPs)

Nanoparticles are organic or inorganic particles in the nanosize range (10^{-9}m) that display unique electronic, optical and magnetic properties due to their small size and surface characteristics (Wahajuddin & Arora, 2012). These properties enable them to be specifically controlled and targeted to the biological component of interest and interact on a cellular, subcellular, protein or genetic level (Pankhurst *et al*., 2003; Krishnan, 2010). Some examples of nanoparticles include MNPs, liposomes, polyplexes, quantum dots and carbon-based nanoparticles (Guo & Huang, 2012; Lian *et al.*, 2014). Nanoparticles have exhibited a wide range of biomedical applications that include cell isolation and imaging, gene/drug delivery, diagnostics and magnetic hyperthermia (Pankhurst *et al*., 2003; Banerjee *et al.*, 2010; Krishnan, 2010).

Magnetic nanoparticles (Figure 1.12) can serve as multifunctional engineering platforms which can be harnessed to meet many technical requirements (Marszall, 2011). Their applications in drug delivery, diagnostics combined with therapeutics such as magnetic resonance imaging (MRI)-guided stem cells are well documented (Cores *et al.*, 2015). The

term magnetic particle refers to a variety of synthetic particles or aggregates of micro- or nanometer size, with distinct physicochemical properties, all sharing a magnetic component (Plank *et al.*, 2003; McBain *et al.*, 2008a). There are many magnetic materials that could be used as the magnetic nanoparticle core such as iron, nickel, cobalt or chromium, but all excluding iron have been found highly toxic for the cells eliminating their use for biomedical applications (McBain *et al.*, 2008a; Berry, 2009; Hofmann-Amtenbrink, 2010). The magnetic cores are coated with a variety of polymers as described in section 1.5.2.2, in order to improve handling and be surface-functionalized in terms of charge and binding capacity of biomolecules and proteins, facilitating gene delivery (Sokolova & Epple, 2008; Yiu, 2011; Lian *et al.*, 2014). The biocompatible polymers serve also the essential purpose of minimizing toxicity preventing the direct contact between cells and magnetic core (Hughes *et al.*, 2005; Kievit & Zhang, 2011; Dizaj *et al.*, 2014).

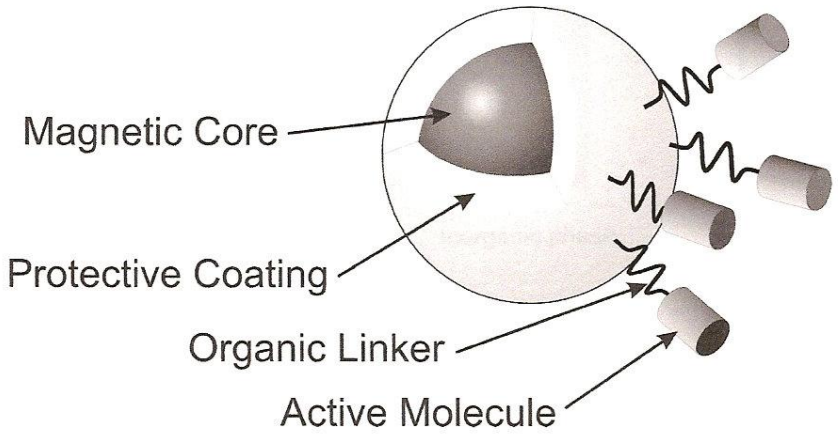


Figure 1.12: Schematic representation of a typical magnetic nanoparticle for biotechnology. The MNP consists of a magnetic core that is coated with a protective layer such as polymer and is surface-functionalized with an organic linker and an active molecule such a protein (McBain *et al*., 2008a).

1.6.3 Superparamagnetic iron oxide nanoparticles (SPIONs)

Superparamagnetic iron oxide nanoparticles constitute a special category of MNPs. They are small synthetic particles coated with a charged polymer, with magnetic cores composed of clusters of magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) crystallites which are ferromagnetic in nature and permanently magnetized (Jiles, 2015). When these materials remain smaller than 30 nm they exhibit their superparamagnetic characteristics described in section 1.6.1, and retain their strong magnetization only in the presence of a magnetic field, being suitable for magnetically guided targeted delivery (Peng *et al.*, 2008; Kievit & Zhang, 2011; Mahmoudi *et al.*, 2011). When the magnetic field is removed the magnetization is lost. This characteristic is desirable for gene/drug delivery vesicles allowing SPIONs to be remotely manipulated by external fields (including MRI), but preventing particle agglomeration and evading uptake by phagocytes while also increasing their half-life in the circulation. Additionally, due to their negligible tendency of aggregation the risk of thrombosis within the vasculature is minimised (Dobson 2008; Hofmann-Amtenbrink, 2010; Wahajuddin & Arora, 2012).

SPIONs can be coated with biocompatible polymers and surface-functionalized as MNPs described previously, or precipitated through a larger porous polymer generating larger particles with superparamagnetic properties (Cortajarena *et al.*, 2014). In the field of medicine and biology, superparamagnets are broadly classified by size were SPIONs range between 50-100 nm, ultra small SPIONs between 10-50 nm and very small SPIONs are <10 nm (Singh *et al.*, 2010; Cortajarena *et al.*, 2014). SPIONs have been well studied for biomedical applications such as gene/drug delivery, medical imaging and regenerative

medicine (Lin *et al.*, 2008; Naqvi *et al.*, 2010; Mahmoudi *et al.*, 2011; Tassa *et al.*, 2011; Thu *et al.*, 2012; Cores *et al.*, 2015). Particles ranging between 10-100 nm are considered optimal as they evade reticuloendothelial system having longer circulation times, and are still able to penetrate through small capillaries (Singh *et al.*, 2010; Mahmoudi *et al.*, 2011). There is an extended market of commercially available SPIONs used in the laboratory *in vitro* and *in vivo* (Hofmann-Amtenbrink, 2010), but only a few are FDA approved for clinical use (Cromer Bermen et al., 2011; Jasmin *et al.*, 2011).

In summary, SPIONs have to posses the following characteristics in order to be suitable for biomedical applications (Mahmoudi *et al.*, 2011; Wahajuddin & Arora, 2012).

- Biocompatibility
- Biodegradability
- Low toxicity
- Physiochemically stable for storage and physiological conditions

For the purpose of this thesis two commercially available SPIONs have been used, nTMag and PolyMag, sharing all the advantageous characteristics of SPIONs for gene delivery as described above, with identical physiochemical specifications (shape, size, surface coating) according to their manufacturers. They will be further described in materials and methods in Section 2.3.3.

1.6.3.1 Cellular uptake of SPIONs

Generally, cells internalize SPIONs by endocytosis (Shapiro et al., 2005). It is understood that SPION parameters such as particle size, shape and surface modifications affect the rate of particle internalization, the specific endocytic pathway used by the cell, and the cellular

location that the particle will end up (Singh *et al.*, 2010; Cores *et al.*, 2015). The manipulation of particle surface using polymer coatings, antibodies and peptides can increase the rate of internalization (Cortajarena *et al.*, 2014). There are several endocytic uptake pathways shown in Figure 1.13, such as: (i) macropinocytosis for particles <5 μm, (ii) micropinocytosis that includes clathrin-mediated endocytosis for particles <300 nm and caveolae-mediated endocytosis for particles <80 nm, and (iii) phagocytosis for particles up to 20 μm (Gu et al., 2011; Canton & Battaglia, 2012).

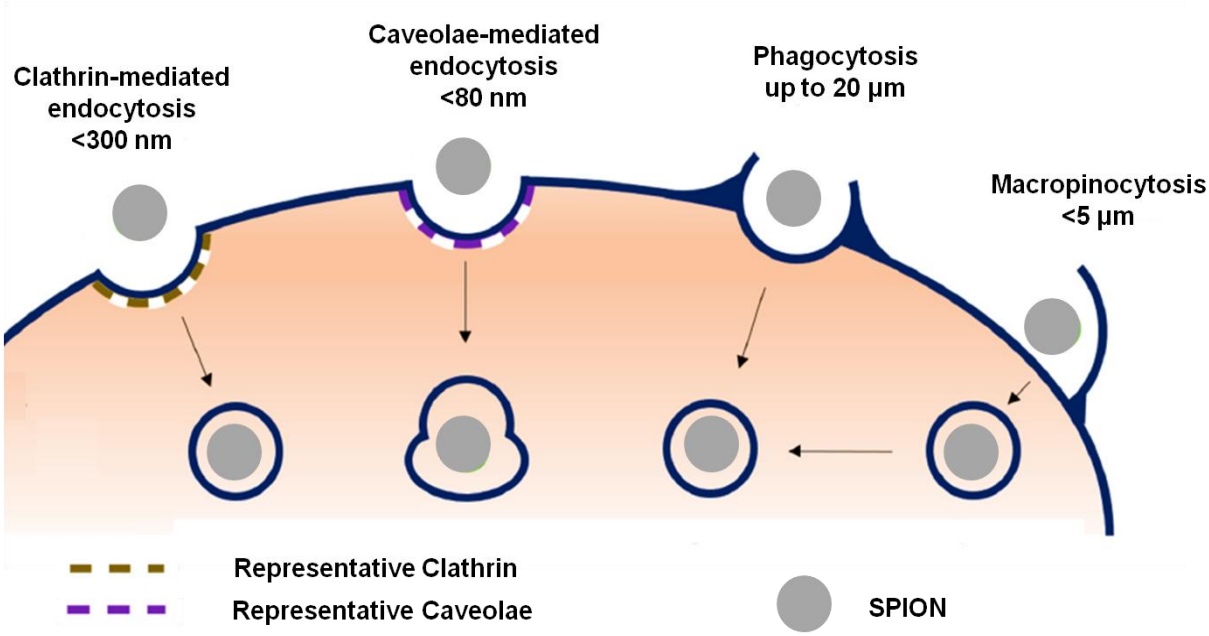


Figure 1.13: Schematic representation of available endocytic pathways for SPION cellular uptake. (Adapted from Cores *et al.*, 2015).

1.6.3.2 Toxicity and clearance of SPIONs

Generally, no acute toxicity of SPIONs has been reported and are considered as inert and biocompatible due to the naturally occurring iron (ferritin) in the human body (Chen *et al.*,

2010; Markides *et al.*, 2012; Cores *et al.*, 2015). The body is adapted for iron metabolism with SPIONs having a good safety profile and some being FDA approved for clinical translation, for use as contrast agents (Schafer *et al.*, 2010), for the treatment for iron-deficiency anaemia (Rosner & Auerbach, 2011) and for tumour therapy with magnetic hyperthermia (Johannsen *et al.*, 2010).

However, at high quantities or concentrations and at various oxidative states iron can be damaging for the cells affecting cell viability and morphological changes in DNA and proteins (Kim *et al.*, 2010; Wang *et al.*2011). Iron exists in the two main oxidative states of Fe (II) and Fe (III), with Fe (II) being responsible for free radical generation and consequently cellular damage (Markides *et al.*, 2012). The imbalance between damaging oxidants known as reactive oxygen species (ROS) causes oxidative stress, which is considered as the main reason of toxicity in MNPs (Kim *et al.*, 2010). Furthermore, SPION physiochemical characteristics such as size, shape and surface properties hold an important role in a potentially toxic response causing aggregates and coagulation which are shape- and size-dependent (Wahajuddin & Arora, 2012). It is clear that there is a need for extensive SPION testing in primary cells and relevant models, followed by pre-clinical animal studies in order to reach clinical trials.

In terms of SPIONs elimination from biological systems, **both metabolism and excretion are to be considered**. Once internalized, degradation of SPIONs is mediated by lysosomes, as generally considered the primary intracellular metabolic mechanism (Levy *et al.*, 2010). Similarly once internalized, three more mechanisms for the metabolic fate of SPIONs have been proposed by a study in macrophage-like mouse cells suggesting that (i) macrophages can eliminate SPIONs during mitotic cell division and passage of those to the

daughter cells; (ii) low lysosomal pH can induce SPIONs degradation and release of Fe (III) in the intracellular medium that can be stored in the body reserves assisted by iron-regulating proteins such as ferritin; (iii) SPIONs could be exocytosed even though limited evidence is available (Gu *et al.*, 2011; Wahajuddin & Arora, 2012).

In terms of excretion the kidney route is considered the most desirable involving minimal intracellular catabolism and reducing the potential of reactive oxygen species generation and therefore associated toxicity (Wahajuddin & Arora, 2012). The safest route is via renal excretion, however the shape and size of particles affects significantly the route of clearance. More specifically regarding the particle shape, it was shown that long rod-shaped particles which have a longer blood circulation time were distributed in the spleen, whereas short rod-shaped and spherical particles were distributed in the liver showing rapid clearance via urine and feces (Huang *et al.*, 2011). Regarding the particle size which determines their half-life circulation, particles <10 nm are removed by renal clearance while particles >200 nm accumulate in the spleen or taken up by phagocytic cells leading to decreased plasma concentrations (Chouly *et al.*, 1996; Wahajuddin & Arora, 2012). As mentioned previously, particles in the range of 10 to 100 nm are considered optimal as they evade reticuloendothelial system and their circulation time is longer (Singh *et al.*, 2010; Mahmoudi *et al.*, 2011).

1.7 Magnetic drug delivery as the basis for MNP-based gene transfection and targeting

1.7.1 The principle of MNP-based drug delivery

Magnetic micro- and nanoparticle-based drug delivery is a chemical and physical combination that offers potential for *in vitro* and *in vivo* gene delivery (Dobson, 2008). Coated and functionalized MNPs can be used as gene carriers and targeted to specific sites *in vitro* and *in vivo* by the application of external, focused magnetic fields (Scherer *et al.*, 2002; Corchero & Villaverde, 2009; Polyak *et al.*, 2009; Lian *et al.*, 2014), forming the basis of magnetic nanoparticle-based gene transfection and targeting. In addition, since systemic drug delivery requires larger doses of circulating drug with associated deleterious side effects in healthy cells, the potential of specific magnetically targeted drug and gene delivery using lower amounts of the therapeutic agent concentrated at the target tissue of interest attracts growing interest (Pankhurst *et al*., 2003; Peng *et al.*, 2008; Corchero & Villaverde, 2009; Pankhurst *et al*., 2009).

Magnetic particles are capable of coupling and responding to an applied magnetic field. In order to generate a force on the magnetic particle the field much have a gradient, as in the presence of a homogeneous field the particle will experience no force (Pankhurst *et al.*, 2003). This is the reason that high-gradient, rare-earth magnets are frequently used for magnetic drug delivery and MNP-based gene transfection purposes (Dobson *et al.*, 2006).
In magnetically targeted therapy *in vivo*, a cytotoxic drug or a gene is bound to biocompatible magnetic micro- or nanoparticles, which then are injected into the patient's blood stream or, in some cases, directly into the target tissue. By the use of externally

applied magnetic fields the drug-particle complexes can be pulled from circulation and targeted to a region within the body or held in place at the target (Figure 1.14) (Pankhurst *et al*., 2003). Subsequently, the therapeutic agent is slowly released from the magnetic carrier by heat, enzymatic activity or degradation of the linker compounds, and a local effect of the agent released is initiated (Nishijima *et al*., 2007; Guo & Huang, 2012).

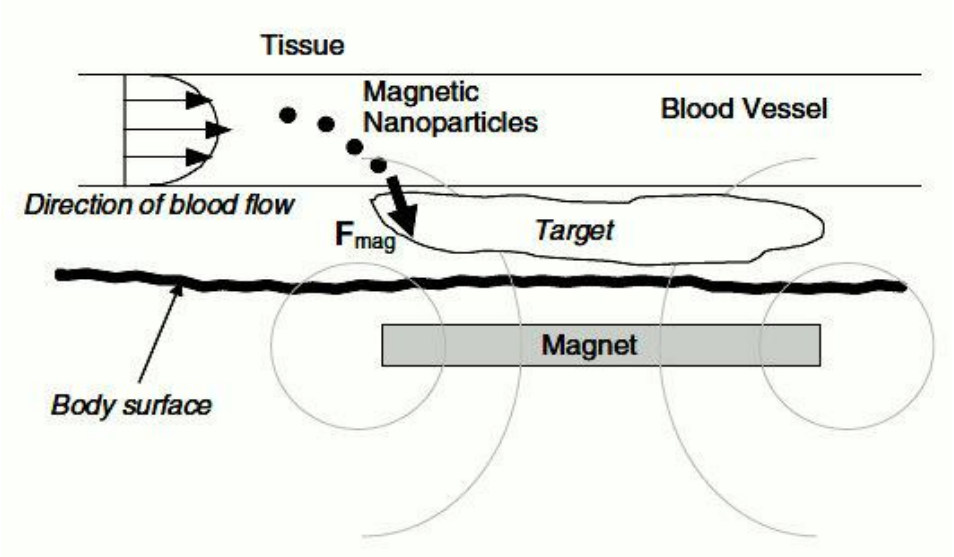


Figure 1.14 Schematic side view section of magnetic nanoparticle-based gene targeting *in vivo*. Gray rings represent the lines of magnetic flux due to externally applied magnetic field. F_{mag} represents the magnetic force vector applied on the particles as they flow through the bloodstream (Dobson, 2006a).

1.7.2 Clinical applications of MNP-based drug delivery

The first clinical application of MNPs was demonstrated in 1957 by Gilchrist *et al* when maghemite (γFe_2O_3) particles were injected and selectively heated using an alternative current (AC) magnetic field to treat lymph nodes and metastases (Gilchist *et al*., 1957). The principle of magnetically guiding particles into specific target areas was introduced in

1963, when intravenously injected iron particles were accumulated into the veins of dog legs by the application of an externally applied magnet (Meyers *et al*., 1963). A few years later, in 1979, the first well defined magnetic microspheres were constructed for the purpose of magnetic targeting (Widder *et al.*, 1979). Following on, several groups have successfully applied magnetically targeted cytotoxic drug delivery in multiple small animal studies (Pankhurst *et al.*, 2003) including swine (Goodwin *et al.*, 2001), rabbits (Alexiou *et al.*, 2000) and rats (Pulfer *et al.*, 1999; Lubbe *et al.*, 2001).

Although this approach has been successfully used in many studies the clinical potential of the method has not yet been realized, with a small number of clinical trials up to date.
In the first clinical trial, epirubicine was complexed with nanoparticles to target advanced solid liver tumours in 14 patients, with effective targeting to the tumour site for 6 patients. Additionally, the particles that were not attracted to the tumour site were accumulated in the liver with no abnormal effects (Lubbe *et al.*1996a; Lubbe *et al.*1996b). In a second clinical trial, doxorubicin hydrochloride was coupled to magnetic particle carrier and delivered intra arterially to the tumour site in 30 out of 32 patients with hepatocellular carcinoma. In the analysis of the first 17 patients, 15 of them showed stable or decreased tumour size (Koda *et al.*, 2002). In a third similar clinical trial, with 4 patients with hepatocellular carcinoma, doxorubicin was coupled to magnetic particle carrier and delivered via the hepatic artery using MRI simultaneously. The particle/doxorubicin complex was well focused, inducing a tumour volume decrease ranging between 64% to 91% (Wilson *et al.*, 2004). Finally, additional data from clical trials in Phase I and II reported targeted intra-arterial hepatic delivery and low cytotoxicity levels of doxorubicin-

transdrug® via biodegradable magnetic polycyanoacrylate nanospheres (Merle *et al.*, 2006; Mody *et al.*, 2014).

1.7.3 The choice of magnetic carrier and targeted delivery through enhanced permeability and retention (EPR)

Despite several successful applications of MNP-based drug delivery *in vitro* and *in vivo*, there are technical barriers that need to be addressed such as the rapid decrease of magnetic field strength when the target tissue is deep into the body and limitations of bypassing intervening vasculature (Dobson, 2006a; Dobson, 2006b; Pankhurst *et al*., 2009;). In order to minimize these limitations and enhance attachment of the drugs, numerous studies have worked on the development of novel MNPs as described in section 1.6.2 that vary in size, multifunctional coatings, magnetic properties, which use high-moment materials and thermoresponsive hydrogels and/or particles (Corchero & Villaverde, 2009; Nishijima *et al*., 2007; McBain *et al*., 2008a; Muthana *et al.*, 2008; Sanvicens & Marco, 2008; Staniland *et al.*, 2009; Staniland, 2009; Xie *et al*., 2010).

For example, particles ranging between 10-100 nm have been reported as optimal, as they have longer circulation times and are still able to penetrate through small capillaries (Singh *et al.*, 2010; Mahmoudi *et al.*, 2011). Such particle sizes allow for increased MNPs concentrations along with macromolecules, to be easier transported at the tumour target site and across the tumour endothelium, due to the tumour's characteristic enhanced permeability and retention (EPR) effect with leaky vascular structures and poor lymphatic drainage (Figure 1.15) (Brannon-Peppas & Blanchette, 2004; Estelrich *et al.*, 2015). For most human leaky tumours, particles with a mean size of approximately 100 nm are

attractive for tumour targeting and allow easier surface modifications to conformations that prevent serum opsonisation, in contrast with larger particles of approximately 400 nm that cannot easily enter the capillary gaps in tumour vasculature (Zhang *et al.*, 2012).

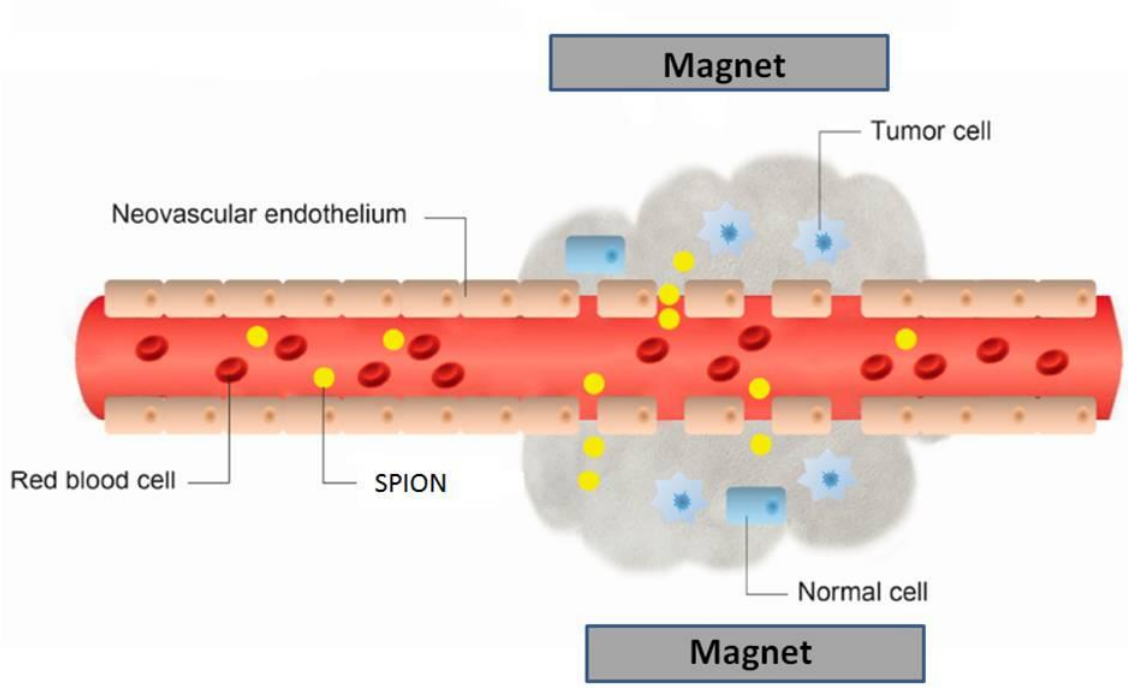


Figure 1.15 Schematic representation of magnetic nanoparticle-based drug targeting in tumour cells *in vivo*, highlighting enhanced permeability and retention effect. The externally applied magnetic field close to the target tissue promotes SPIONS localization at the tumour site, and via blood circulation around and/or inside the tumour endothelium, SPIONS extravasate from pores of leaky tumour blood vessel walls reaching the tumour site, a characteristic known as EPR effect (Adapted from Estelrich *et al.*, 2015).

In summary, magnetic drug targeting must be safe and effective, and be capable of delivering the maximum amount of therapeutic agent with the least possible amount of

magnetic particles (Corchero & Villaverde, 2009; Pankhurst *et al*., 2009). Additionally, the most desirable magnetic carriers possess characteristics such as (Wahajuddin & Arora, 2012);

- Small size to allow insertion into the target area
- A wide range of particle sizes to allow their selection depending on the specific application
- A large biocompatible surface area and high capacity for transgene or therapeutic agent uptake
- The ability to biodegrade, eliminating toxic by-products.

Based on the MNP-based gene transfection principles explained here for magnetic drug/gene delivery and targeting purposes, the novel non-viral nanomagnetic gene transfection method is presented next.

1.8 MNP-based gene transfection: Nanomagnetic transfection

1.8.1 Overview of principles and key parameters for nanomagnetic transfection

Nanomagnetic gene transfection follows on from the general principle of magnetically directed gene delivery presented in section 1.7.1. As briefly introduced in Figure 1.4 about gene delivery approaches and Section 1.5.3 about hybrid systems, it combines both physical and chemical characteristics and has been effectively used with both viral and non-viral vector systems (Mah *et al.,* 2000; Dobson *et al.,* 2006; McBain *et al.,* 2008b). Our group focuses on the technique that involves the coupling of MNPs with reporter or therapeutic genes (DNA or siRNA) to form complexes, which are applied in mammalian cell cultures and are taken up by endocytosis. Endocytosis is promoted further by the presence of a novel externally introduced oscillating magnet to assist and direct specific delivery of MNP/gene complexes on a single cell basis (Section 1.8.3) (McBain *et al.*, 2008b; Lim *et al.*, 2012).

In terms of the method's background, MNP-based gene transfection (Figure 1.16), as the principle of nanomagnetic transfection, was introduced in 2000 by Mah *et al.* who first demonstrated the use of magnetic microparticles for gene transfection purposes and linked those to AAV encoding Green Fluorescent Protein (GFP) reporter gene. The complexes were magnetically targeted by static high-strength, high-gradient neodymium iron boron (NdFeB) magnets to a specific region of C12S HeLa-derived cells *in vitro* and in mouse models *in vivo* following intramuscular injection (May *et al*., 2000; May *et al.*, 2002). The magnetic targeting enabled highly efficient uptake of GFP gene by HeLa cells localized at the site of the applied magnetic field. The method has generated great interest and based on

this idea many subsequent studies have applied the principle to non-viral systems that will be discussed in the following sections 1.8.2 and 1.8.3.

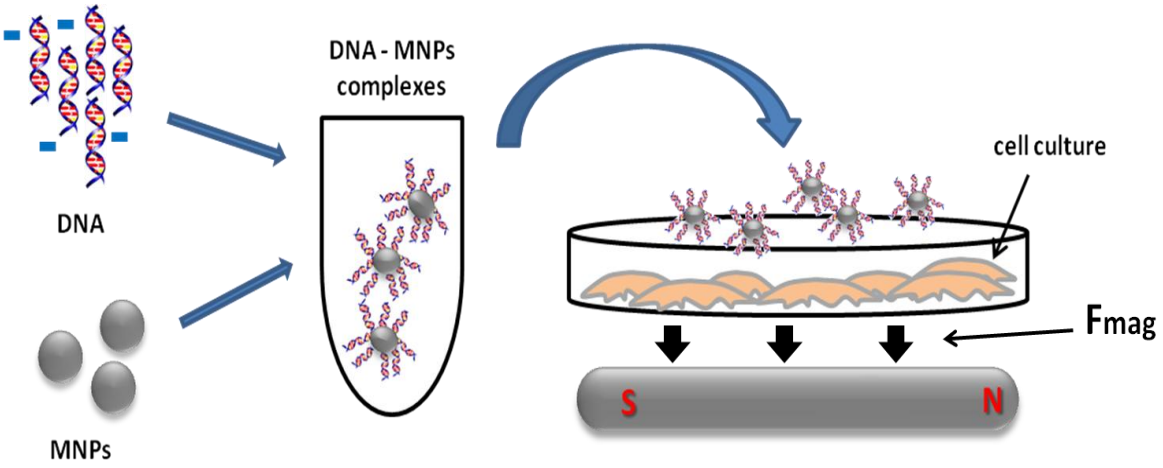


Figure 1.16 Schematic representation of MNP-based gene transfection *in vitro*. The vector (here DNA) is attached to MNPs to form complexes, which are added to the cell culture. The high-gradient, rare-earth magnet is placed below the culture dish, and pulls the particles towards the magnetic field source. F_{mag} is the force exerted on the particles by the magnetic field.

For nanomagnetic gene transfection, naturally the presence of a magnetic field and the choice of the MNPs to be used are of essential importance and these parameters need to be considered as part of the optimization of transfection efficiency.

In regards to the **magnetic field,** during *in vitro* nanomagnetic transfection MNP/gene complexes are introduced into the cell culture, where the required field gradient is produced by the externally applied rare-earth magnets. Generally, neodymium iron boron (NdFeB) magnets are used and placed directly underneath the cell culture. The main advantage of this system is the ability to increase the speed of particle sedimentation onto

the cell surface and therefore significantly reduce the required time for cell transfection (Figure 1.16) (Plank *et al*., 2003; Dobson, 2006a; Fallini *et al.*, 2010). When SPIONs are used for nanomagnetic transfection, the fundamental mechanism by which the system works is determined by the equation:

$$F_{mag} = (\chi_2 - \chi_1) \times V \times 1/\mu_0 \times B \times (\nabla B)$$

Where, F_{mag} is the force on the magnetic particle, χ_2 is the volume magnetic susceptibility of the magnetic particle, χ_1 is the volume of magnetic susceptibility of the surrounding medium, μ_0 is the magnetic permeability of free space, V is particle volume, B is the magnetic flux density in Tesla (T), ∇B is field gradient and can be reduced to $\partial B/\partial x$, $\partial B/\partial y$, $\partial B/\partial z$, of which $\partial B/\partial z$ plays the most critical role due to the geometry of the system (Pankhurst *et al.*, 2003). This results in a translational force on the MNP:gene complex in the direction of the magnetic field source, placed underneath the cell culture.

For *in vivo* applications, following the magnetically targeted gene delivery model illustrated in the previous section 1.7.1 (Figure 1.14 and 1.15), the particles carrying the therapeutic gene could be intravenously injected and circulated by the blood stream until captured by externally applied magnets. These magnets could be focused close to the target tissue, to promote transfection and targeting of therapeutic genes to a specific site or organ of the body.

In regards to the **MNPs,** the selection of the appropriate type, size and surface coating attributes some essential properties to the magnetic carrier/gene complexes (Section 1.6.2): (i) the use of SPIONS for their advantageous superparamagnetic properties provides strong magnetization for rapid complex sedimentation, which is lost once the magnetic field is

removed, preventing particle aggregation *in vitro* and *in vivo* (Sections 1.6.1 and 1.6.3) (Dobson *et al.,* 2007; Ortega, 2012). (ii) the size of SPIONS in the range of 100 nm is advantageous for penetrating through small capillaries (Mahmoudi *et al.,* 2011), and transfer of the MNP:gene complexes into the cells via a number of endocytic pathways (Section 1.6.3.1).

(iii) the surface coating of MNPs (including SPIONS) is responsible for effective binding with plasmid DNA or siRNA, surface functionalization facilitating gene delivery, and minimizes toxicity once in contact with the target cells (Sections 1.5.2.2, 1.6.3 and 1.6.3.2). Additionally, the choice of a charged polymer for the particle coating such as PEI promotes endosomal escape, as it allows exploitation of the changes in pH which occur within the internalized endosome activating the proton sponge effect mechanism (Figure 1.9) with endosomes rupture, and release of the MNP:gene complex into the cell's cytoplasm(Section 1.5.2.2) (Demeneix & Behr, 2005; Akinc *et al.,* 2005; Midoux *et al.*, 2009; Richard *et al*., 2013).

MNPs development is a continuous process and their investigation in tayloring the many requirements for transfection purposes has been reported in numerous studies. Several reviews have been published summarizing their current status (Mahmoudi *et al.,* 2011; Markides *et al.,* 2012; Prodan *et al.,* 2013; Estelrich *et al.,* 2015).

In general, in order to avoid attachment with other external elements MNPs can be spread into a polymer matrix such as silica or can be enclosed within a polymer or metallic coat (Plank *et al*., 2003). Other particles such as magnetoliposomes are similar to liposomes with a magnetic ferrite core structure enclosed within a spherical lipid membrane (Gonzales & Kirshnan, 2005). Also, mesoporous silica nanoparticles which may consist of

up to 80% iron oxide have been used along with several molecules to promote uptake by the target cells and have been associated with N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP chloride) to both prevent particle aggregation and promote uptake (Yiu *et al*., 2005; Dobson, 2006a;). A comparison between small (30-60nm) and large (300-600nm) nanoparticles has shown that both types were transfected but the large nanoparticles were able to achieve higher transfection efficiencies, highlighting the importance of the size of the MNP/DNA complex for transfection efficiency (Schillinger *et al*., 2005).

Once the MNP formation and consistency have been optimized, the coupling to the corresponding gene of interest follows. The gene of interest may be linked to naked DNA or enclosed into a vector. MNPs can be linked to their gene of interest via a viral or non-viral vector and coupled through both electrostatic interactions and salt induced colloid aggregation as well as cross-linking (Plank *et al*., 2003; Agora *et al.,* 2013). Their therapeutic genes can be released by enzymatic cleavage of cross-linking molecules, pH charge interactions and polymer matrix degradation (Dobson, 2006a; You & Auguste, 2010). For several types of MNPs that can be embedded within a matrix such as a hydrogel, the release of the therapeutic gene is mediated by heating of the hydrogel carrier (Pankhurst *et al*., 2009).

1.8.2 Nanomagnetic transfection studies using static magnet arrays *in vitro* and *in vivo*

Over the past decade, and based on the idea of MNP-based gene transfection as presented by Mah *et al.* in the previous section, significant progress has been made on further developing the technique by Plank, Rosenecker and others that coined the term 'magnetofection'. In this group's studies magnetofection using static magnet arrays and therefore static magnetic fields, was used to importantly demonstrate the potential of the technique for non-viral transfection *in vitro* and *in vivo* in a variety of cell types using genetic material coupled to MNPs and/ or SPIONs with a magnetite core and an organic or inorganic coating (Scherer *et al*., 2002; Plank *et al*., 2003; Schillinger *et al*., 2005; Neuberger *et al*., 2005; Mykhaylyk *et al*., 2008).More specifically, Scherer *et al.* first used iron oxide nanoparticles coated with PEI associated with DNA vectors by electrostatic interaction, to show increased transfection efficiency in a number of transfection reagents *in vitro* (Scherer *et al*., 2002). In addition, reduction of tranfection duration was achieved due to the principal advantage of MNP-based gene transfection, the rapid sedimentation of MNP/gene complex onto the target area. Furthermore, the transfection efficiency obtained *in vitro* was reported similarly *in vivo* with magnetic field-guided local transfection in the gastrointestinal tract and blood vessels of rats. In the same study, adenoviral vectors were complexed with MNPs that enabled transduction of previously non-permissive cell types, indicating that the host tropism of adenoviral vectors can be extended with MNP-based transfection technologies (Scherer *et al.*, 2002).

Following on from this original study, the method has been used to transfect *in vitro* primary lung epithelial cells and whole tissue samples of airways (Gersting *et al.* 2004), and blood vessel endothelial cells (Krotz, Sohn *et al.*, 2003). MNPs have also been used to

deliver antisense oligonucleotides *in vitro* and siRNA downregulating gene expression *in vivo* (Krotz, de Wit *et al.*, 2003).

In 2005, Schillinger *et al.* reported significant reduction of retrovirally mediated expression of luciferase in Hela cells by siRNA delivery (Schillinger *et al.*, 2005). In the same study, the applicability of the method was tested further with MNPs associated with small and large nucleic acids transfecting *in vitro* primary cells such as keratinocytes, chondrocytes, osteoblasts and amniocytes. Furthermore, the group initiated a veterinary clinical study which reported *in vivo* therapeutic effect in feline fibrosarcoma using MNPs to deliver the gene encoding for human GM-CSF (granulocyte macrophage colony stimulating factor). In these experiments, an externally positioned magnet was fixed on the tumour in order to induce vector localization at the site of interest (Schillinger *et al.*, 2005).

In 2008, Mykhaylyk *et al*., using MNPs reported increased *in vitro* siRNA delivery in adherent mammalian cells compared with cationic lipids (Mykhaylyk *et al*., 2008). Two years later, the method was used to demonstrate efficient transfection of primary motor neurons (Fallini *et al.*, 2010). This group has shown for the first time that the spinal muscular atrophy-disease protein Smn is actively transported along axons of live primary motor neurons, and that gene knockdown data using small hairpin (sh)RNA-based constructs showed significantly reduced Smn levels in both cell bodies and axons (Fallini *et al.*, 2010). Following on, siRNA was successfully delivered in primary and immortalized mammalian fibroblasts and the method was compared with lipid-mediated delivery. The data reported high transfection efficiencies with both techniques and a gene silencing effect of approximately 80%. However, MNP-based transfection showed significantly lower cytotoxic effect post-transfection (Ensenauer *et al.*, 2011).

More recently, Amin, Stevens *et al.*, showed stimulation of chondrogenic differentiaton of adult human bone marrow-derived stromal cells following a 14-day application of a static magnet field. This work highlighted the importance of inducing chondrogenesis in precursor cells as it could benefit cases of Osteoarthritis (Amin *et al.*, 2014). In 2016, Brett *et al.*, used a prefabricated scaffold integrated with PEI-coated MNPs complexed with a plasmid encoding GFP and B-cell lymphoma 2 (Bcl-2) for *in vivo* transfection in mice with external magnetic field. It was demonstrated upregulation of Bcl-2 in implanted human adipose-derived stromal cells and bone regeneration enhancement (Brett *et al.*, 2016). There was a comparison between magnetofection and nucleofection to reveal significantly more healing in the magnetofected groups.

It is clear that MNP-based gene transfection has been used widely, and nanomagnetic transfection using static magnet fields, is a rapid and efficient non-viral technique for transfection *in vitro* with promising *in vivo* data to this day. However, the need for further investigating the method remains, in order to improve further transfection efficiency and/ or extend its potential and use in a wider range of biomedical applications.

1.8.3 Nanomagnetic transfection studies using oscillating magnet arrays *in vitro* and *in vivo*: Recent advancements

In 2005, our group first introduced work on the oscillating magnetic fields in the x-y plane (Dobson, 2005; Dobson & Batich, 2005). Since then, a lot of research has been focused on the optimization of the technique by the use of oscillating magnet arrays, to enhance overall transfection efficiency of magnetofection-type approaches using static magnets and further decrease transfection duration, while maintaining the high cell viability that nanomagnetic gene transfection offers.

In this novel system, MNP/gene complexes are added into the cell culture in the presence of a horizontally oscillating magnet array positioned beneath the cell culture plate, which introduces a lateral component to the motion of the MNP/gene complexes, further promoting endocytosis. The complexes are uptaken by the cells, often via clathrin-mediated endocytosis when SPIONs are used, while generally this depends on the size of the MNPs, as described in section 1.6.3.1 (Figure 13) (Schillinger *et al.*, 2005; Cores *et al.*, 2015). Once into the cytoplasm, the buffering capacity of the polymer coated MNPs (here PEI) induces the proton sponge effect, rupturing the endosome and releasing the DNA (in case of DNA transfection) as decribed in section 1.5.2.2 (Figure 1.9). Then, DNA nuclear transportation follows for the transcription of the target protein (Figure 1.17) (Fouriki *et al.*,2010; Restani *et al.*, 2014).

In regards to the mechanism and effect of the oscillating magnetic field; although MNPs will generally experience torque in a homogenous magnetic field, they will also undergo translational movement when exposed to a field gradient, which will be further influenced

by the magnet array horizontal displacement (Pankhurst *et al.*, 2003). This lateral motion of the MNP/gene complexes is primarily perpendicular to the translational force exerted on the particles by the field gradient, which adds energy and mechanical stimulation to the system (Dobson, 2006; McBain *et al.*, 2008a). Based on the above, we hypothesize that the energy and mechanical stimulation introduced onto the cell membrane by the MNP/gene complexes moving in the x-y plane under the influence of the oscillating magnet array, further promote MNP/gene complexes uptake by the cells improving transfection efficiency (McBain *et al.*, 2008b; Fouriki *et al.*, 2010; Lim & Dobson, 2012).

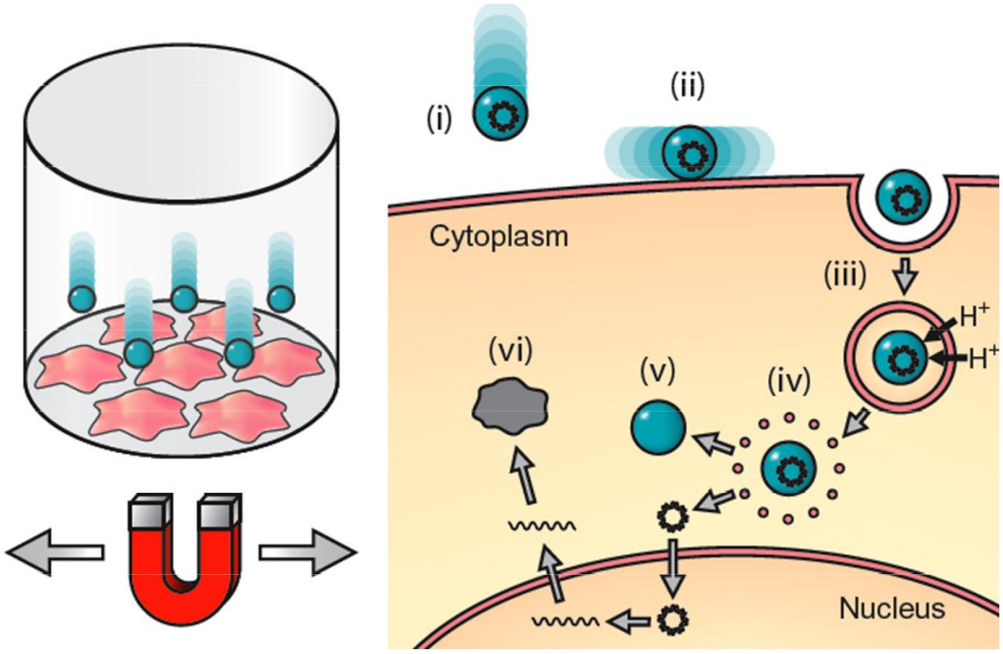


Figure 1.17-: Principle of oscillating nanomagnetic transfection. Plasmid DNA or siRNA is attached to magnetic nanoparticles and incubated with cells in culture (left). An oscillating magnet array below the surface of the cell culture plate pulls the particles into contact with the cell membrane (i) and drags the particles from side-to-side across the cells (ii), mechanically stimulating endocytosis (iii). Once the MNP/DNA complex is

endocytosed, proton sponge effects rupture the endosome (iv) releasing the DNA (v) which then transcribes the target protein (vi) (Fouriki *et al.,* 2010).

The concept of enhanced translocation of particles across the cell membrane stimulated by pulsating electromagnets with increased transfection efficiency was also presented in 2006 by Kamau *et al.*, although significant heating effect was produced requiring weaker field strengths and gradients (Kamau *et al.,* 2006). Such heating effects in combination with an incubator's conditions during transfection that could contribute additional heat to the cells, are not alarming with the use of the nanomagnetic oscillating system, as permanent high field/gradient magnets are used which hold major advantages in comparison to electromagnets (McBain *et al.,* 2008b). The retention of excellent cell viability levels, comparable to untransfected controls, has been reported by all researchers work used the method and will be described next (McBain et al. 2008b; Fouriki *et al.* 2010; Pickard & Chari, 2010; Jenkins *et al.*, 2011; Fouriki *et al.*, 2012; Lim *et al.*,2012; Lim & Dobson, 2012; Adams *et al.*, 2013; Fouriki & Dobson, 2013; Jenkins *et al.*, 2013; Subramanian *et al.*, 2013; Fouriki *et al.*, 2014; Subramanian *et al.*, 2017)

The additional energy transferred from the oscillating field to the cells, promotes particle uptake and apart from transfection efficiency also increases protein expression levels as demonstrated in the following studies.

In 2008, McBain *et al.*, used iron oxide nanoparticles coated with PEI carrying the luciferase reporter gene for *in vitro* transfection of human airway epithelial cells. It was shown that at 2 hrs duration, a statistically significant increase in overall transfection efficiency in response to an oscillating magnet array, was two times greater compared to

both static field and four times greater that two different cationic lipids tested. Cell viability levels were similar to controls at 48 hrs post transfection indicating that the method was not harmful to the cells (McBain *et al.*, 2008b).

In 2010, using the same cell type and PEI coated SPIONS carrying the luciferase reporter gene, protein production was further investigated *in vitro.* It was observed enhanced transfection efficiency and by up to tenfold increase in protein production, in comparison to the use of both static field nanomagnetic gene transfection, as well as the most widely used cationic lipid-based technique, with no adverse effects on cell viability (Fouriki *et al.*, 2010). The above two initial studies by our group showed that transfection efficieny was also dependent on the magnetic field gradient, frequency and amplitude, with 40 mT, 2 Hz and 0.2 mm being the optimal for NCI-H292 cells.

In addition, further experimental work has repeatedly demonstrated *in vitro* that nanomagnetic transfection using the oscillating magnet array can efficiently transfect a variety of cell systems, at shorter transfection times (usually 30 minutes) utilizing lower amounts of DNA than cationic lipid-based agents when used for comparison, showing no adverse effects in cell viability and morphology.

In 2010, hard-to-transfect rat astrocytes were efficiently transfected with SPIONs and reporter gene, using the oscillating system that was shown to be frequency dependent. Following introduction of transfected astrocytes into a 3D neural tissue array, cells survived and were able to differentiate (Pickard & Chari, 2010).

In 2011, it was shown that nanomagnetic transfection can safely mediate single and/or combinatorial *in vitro* gene delivery to nuclear precursor/stem cells. Combinatorial gene transfection significantly enhanced overall transfection with negligible toxicity, while no adverse effects were observed on stem cell proliferation and differentiation. The

transfected nuclear precursor/stem cells survived and differentiated in 3D neural tissue arrays post-transplantation (Pickard *et al.*, 2011). In the same year, primary rat oligodendrocyte precursors were efficiently transfected for the first time with MNPs and reporter gene, with increased levels of transfection compared to lipofection and electroporation and significantly higher cell viability (Jenkins *et al.*, 2011).

In 2012, human osteosarcoma fibroblasts (MG-63s) were transfected with SPIONs carrying green fluorescent protein, showing increased transfection efficieny compared to static fields and statistically significant increase in comparison to both cationic lipids and electroporation (these data will be presented as part of this thesis) (Fouriki *et al.*, 2012).

In the same year, using the same nanomagnetic transfection concept, hard-to-transfect mouse embryonic fibroblasts (MEFs) and human umbilical vein endothelial cells (HUVECs), showed significantly higher transfection efficiency compared to both static magnet and lipid reagents (Lim & Dobson, 2012). Also in 2012, for the first time using the method, short interfering RNA (siRNA) was introduced against green fluorescent protein (GFP) or actin into stably-transfected GFP-HeLa cells or wild-type HeLa and rat aortic smooth muscle cells, respectively. This gene silencing technique occurred in a dose- and cell density- dependent manner, as reflected using fluorescence intensity and adhesion assays (Lim *et al.*, 2012).

In 2013, mouse embryonic fibroblasts (NIH3T3s) were transfected with SPIONs and green fluorescent protein, and shown to significantly enhance transfection efficiency compared to cationic lipids (these data will be presented as part of this thesis) (Fouriki & Dobson, 2013). In the same year, human prenatal cardiac progenitor cells and adult cardiomyocytes were transfected with the same nanomagnetic transfection concept, and demonstrated significantly improved transfection efficieny compared to static fields, cationic lipids and

electroporation. Also, it was shown that seeding cells onto Collagen-I was further improved transfection effiency (Subramanian *et al.,* 2013). In another study, investigating for the first time the potential of MNP-mediated reporter gene delivery in oligodendrocytes, it was shown that nanomagnetic transfection using both static and oscillating magnets were achieved, even though transfection efficiency was typically low. This study importantly demonstrated that within cells of a specific neural lineage, the amenability to transfection is dependent on the differentiation status of the cell (Jenkins *et al.,* 2013).

Furthermore, interesting results were demonstrated by Adams *et al.,* when MNPs and reporter gene were used for transfection of neural stem cells propagated in the neurosphere (suspension culture) model. It was shown that transfection efficiency was enhanced over two-fold by oscillating magnetic fields, while there was no effect on cell vialbility, stem cell marker expression and differentiation profile of cultures post transfection. This work demonstrated the first attempt in transfecting suspension cultures of neural cells with MNP-based technology (Adams *et al.,* 2013).

In 2014, human mesenchymal stems were transfected with SPIONs and green flurescent protein, showing increased transfection efficieny compared to both static fields and cationic lipids. In addition, it was observed that hMSC-specific cell surface markers CD44, STRO-1, CD90 and CD146 retained normal expression post transfection (these data will be presented as part of this thesis) (Fouriki & Dobson, 2014).

More recently, efficient transfection of human neuroblastoma cells (SH-SY5Ys) as well as primary hippocampal and cortical neurons which are considered hard-to-transfect neuronal cell types, was demonstrated using oscillating nanomagnetic transfection and MNP/reporter gene complexes. Cell viability was reported high and there was no

interference with the cell physiology, such as differentiation and neurite growth, in both cell lines and primary neurons (Subramanian *et al.*, 2017).

All the above mentioned research works have been tested *in vitro*, showing the successful applicability of the method, but also highlighting the need for *in vivo* testing.

So far, a preliminary *in vivo* work was presented in 2008, where Muthana, Dobson *et al.* trying to improve transfected monocytes' ability to infiltrate tumours, designed a monocyte extravasation assay and demonstrated *in vitro* that transfected human monocytes were able to migrate across a human endothelial cell layer into a 3D tumour spheroid in a significantly increased rate, when the monocytes were pre-loaded with MNPs with the application of a magnetic field close to the tumour spheroid. In the same study, this magnetic targeting approach was applied *in vivo,* and the systemic administration of MNP pre-loaded monocytes in mice tumours demonstrated a marked extravasation increase (Muthana, Dobson *et al.*, 2008).

Ultimately, in future *in vivo* applications, based on the magnetically targeted gene delivery model illustrated in the previous section (Figure 1.14), the particles carrying the therapeutic gene could be intravenously injected and circulated by the blood stream until captured by externally applied magnets at the site of interest. These magnets could be focused close to the target tissue, to promote transfection and targeting of therapeutic genes to a specific site or organ of the body (Dobson, 2006b).

1.9 Stem cells and cell lines used for *in vitro* transfection

In order to meet a primary aim of this work, a variety of mammalian cell types relevant to regenerative medicine was employed for the nanomagnetic gene transfection experiments. In this way, the ability of the method to transfect different cells types was demonstrated and in parallel these results could be used to expand our understanding on how to achieve targeted gene delivery *in vitro* that would ultimately serve for regenerative therapy strategies through gene delivery and cell/tissue engineered-mediated approaches (Daar & Greenwood, 2007). The stems cells and cell lines used in this study are presented in the following paragraphs.

1.9.1 Human mesenchymal stems cells

Human mesenchymal stem cells (hMSCs) were first discovered within the bone marrow from the revolutionary work of Friedenstein and colleagues (Friedenstein *et al*., 1970). They are adult stem cells defined as multipotent, capable of differentiating into a limited number of phenotypes (Lee & Hui, 2006). MSCs are adherent and spindle-shaped, forming colonies in culture which are known as colony forming unit-fibroblasts (CFU-Fs) (Figure 1.18).

MSCs are nonhematopoietic stromal cells that can be ideal candidates for tissue engineering and reparative/regenerative medicine due to their characteristic properties to differentiate and help to regenerate multiple tissues such as bone, cartilage, muscle, ligament, tendon and adipose (Pittenger *et al.*, 1999; Chamberlain *et al.*, 2007; Chen *et al.*, 2008; Schop *et al.*, 2009).

In culture, they are able to divide multiple times and retain their multipotent differentiation characteristics but their identification and characterisation can be challenging due to the lack of specific biomarkers. However, the established guidelines for MSC characterization require the positive expression of CD73, CD90, CD44, CD146, CD105 and STRO-1 antigens (Simmons & Torok-Storb, 1991; Pittenger *et al*., 1999; Colter *et al*., 2001; Delorme *et al*., 2009) and negative expression of CD34, CD45, CD14, CD11b, CD19 or MHC class II antigen and show capability of tri-lineage differentiation into osteoblasts, chondrocytes and adipocytes (Dominici *et al*., 2006; Chamberlain *et al.*, 2007; Chen *et al.*, 2008).

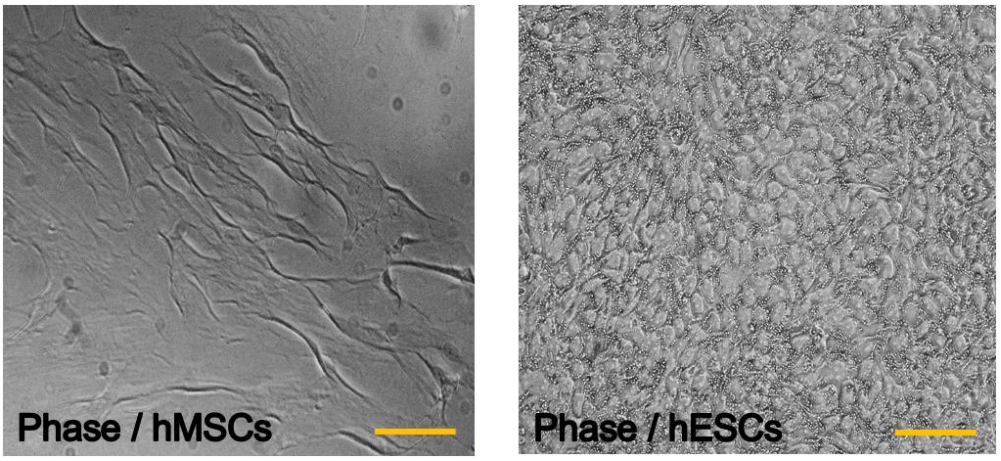


Figure 1.18: Representative phase images of primary cells, hMSCs and hESCs. (A) Human bone marrow derived hMSCs at passage 1, showing the typical fibroblast like morphology of hMSCs. (B) hESCs (SHEF-1 cell line), showing the typical morphology of hESCs expanding as dense and tightly adherent colonies. Scale bar =100μm.

1.9.2 Human embryonic stem cells

Human Embryonic Stem Cells (hESCs) represent perhaps the most important cell type for myriad potential biomedical applications in regenerative medicine, the development and investigation of human disease models, developmental biology studies and many other applications. Human ESCs are known for their immortality as a result of high telomerase activity (Wobus & Boheler, 2005; Thomson *et al.*, 1998) unlimited *in vitro* self-renewal, pluripotency and differentiation capability to generate specialized cell types of three somatic germ layers; ectoderm, mesoderm and endoderm lineages (Gerecht-Nir & Itskovitz-Eldor, 2004).

Human ESCs are derived from the inner cell mass (ICM) of pre-implantation blastocysts and various studies have characterized their undifferentiated phenotype. Their pluripotency is confirmed by the presence of specific surface markers such as SSEA-1, SSEA-4, TRA-1-60, membrane bound receptors such as gp130, the expression of transcription factors such as Oct-4, hTERT, Nanog (Wobus & Boheler, 2005; Cryz *et al.*, 2003; Mathew *el al.*, 2010; Miyazaki *et al.*, 2008) and high levels of alkaline phosphatase activity (ALP) (O'Connor *et al.*, 2008). During *in vitro* culture, hESCs are characterized by a rounded morphology with a large nucleus to cytoplasmic ratio and they expand as dense and tightly adherent colonies that diffuse together to form a confluent monolayer of undifferentiated cells (Figure 1.18) (Thomson *et al.*, 1998).

1.9.3 Established Cell lines

In this study apart from primary human stem cells, three more established cell lines were used as cell model for nanomagnetic gene transfection. Representative images from human osteosarcoma fibroblasts (MG-63), human lung mucoepidermoid carcinoma (NCI-H292) and mouse embryonic fibroblast (NIH-3T3) cell lines are shown below (Figure 1.....).

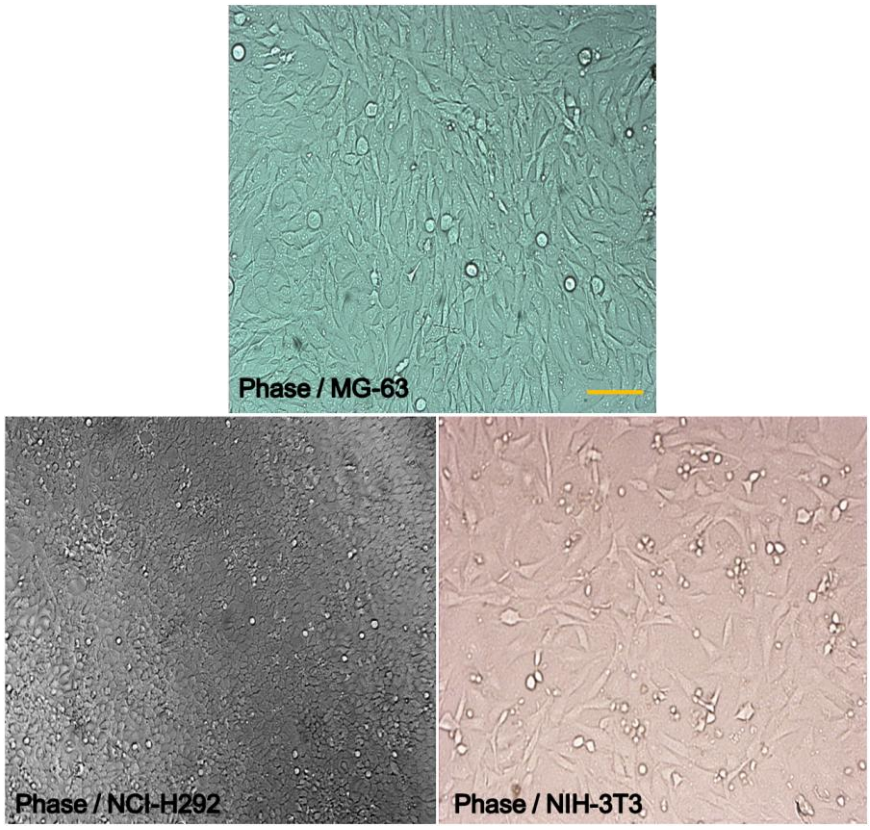


Figure 1.19 Representative phase images of typical morphology of MG-63, NCI-H292 and NIH-3T3 cell lines. Scale bar =100μm.

1.10 Aims and objectives of this study

Gene delivery and its wide potential and uses have been successfully attempted by (a) viral vectors with high transfection efficiency but also significant risks and/or limitations and (b) non-viral vectors comprising of physical and chemical approaches characterized by quite varied results in transfection efficiency and cell viability as presented in this chapter. However, magnetic nanoparticle-based gene transfection systems that generate significant interest in the gene delivery field have shown promise as they can combine considerable transfection efficiency with minimal effects on cell viability eliminating viral associated risks.

Through this study, nanomagnetic gene transfection, as a novel non-viral gene delivery approach has attempted to pursue two main aims:

Aim (1) Efforts have been made to optimize technical parameters of the method in order to further improve the efficiency of reporter gene delivery. At the same time primary cells, hMSCs and hESCs, as well as the cell lines MG-63, NCI-H292 and NIH-3T3 were utilized to assess the improvements in transfection efficiency and to investigate the applicability of the technique in a wider variety of cell types used for regenerative purposes. Furthermore, the outcome of these experiments was not to compromise cell viability, morphology and phenotypic characteristics of these cells following treatment with the nanomagnetic gene transfection system.

Aim (2) In parallel, and in order to assist in the overall evaluation of the method, transfection efficiency and cell viability results obtained by nanomagnetic gene transfection were directly compared with the widely used commercially available lipid-

mediated gene delivery technique Lipofectamine (LF2000) and selectively and when possible with electroporation (AMAXA).

Chapter 2

Materials and Methods

2.1 Cell lines

The human primary cells hMSCs and hESCs (SHEF-1), along with the human NCI-H292, and MG-63, and mouse NIH-3T3 cell lines used for the transfection experiments of this study are listed in the table below (Table 2.1).

Table 2.1: Primary cells and cell lines used for transfection.

Cell lines	Description	Origin
hMSC	Human mesenchymal stem cells isolated from bone marrow aspirates	Human bone marrow aspirates from Lonza, USA
SHEF-1	Human embryonic stem cell line	UK Stem Cell Bank with approval from the Steering Group. In collaboration with Keele University Stem Cell group
MEFs	Mouse embryonic fibroblasts isolated from 12.5-13.5 days gravid uteri of black CB1 hybrid mice	Provided by the Stem Cell group, Keele University small animal facilities
NCI-H292	Human lung mucoepidermoid carcinoma epithelial cell line	ATCC, USA
MG-63	Human osteosarcoma fibroblast cell line	ATCC, USA
NIH-3T3	Mouse embryonic fibroblast cell line	ECACC, UK

2.2 Cell culture

The cell culture materials used for this study including media and other supplements are listed in table below (Table 2.2).

Table 2.2: Media and other supplements for cell culture.

Name	Description	Cat No	Company
DMEM	Dulbeco's Modified Eagle Medium	21969	Gibco, Invitrogen, Paisley, UK
KO-DMEM	Knock Out DMEM	10829	Gibco, Invitrogen, Paisley, UK
FBS	Fetal Bovine Serum	DE14-870F	Lonza, Belgium
KO-SR	Knock Out Serum Replacement	10828	Gibco, Invitrogen, Paisley, UK
PBS	Phosphate Buffered Saline	BE17-516F	Lonza, Belgium
RPMI	Roswell Park Memorial Institute Medium	LM-R1641	Biosera, UK
Trypsin/EDTA	Trypsin 10X with Versene	BE02-007E	Lonza, Belgium
DMSO	Dimethyl Sulphoxide	D2650	Sigma-Aldrich, UK
L-glutamine	L-Glutamine	BE17-605E	Lonza, Belgium
NEAA	Non Essential Amino Acid	BE13-114	Lonza, Belgium
Matrigel	BD Matrigel Matrix	354234	BD, Bedford, MA,

			USA
bFGF	Human FGF-basic	100-18B	PeproTech, London
PSA	Penicillin-Streptomycin-Amphotericin B	17-745E	Lonza, MD, USA
β-mercaptoethanol	2-Mercaptoethanol	31350-010	Gibco, Invitrogen, Auckland, NZ

2.2.1 hMSC culture

hMSC isolation:

Human bone marrow aspirates (BMA) purchased from Lonza, USA were used to isolate from and expand hMSC following the previously published methodology of plastic adherent culture technique (D'Ippolito *et al*. 2004, Wimpenny *et al*. 2010). For this study 2 human BMA from 2 different donors were aspirated from iliac crest and the donors' details were listed in Table 2.3. Whole bone marrow was seeded at a density of 10^5 mononuclear cells/cm^2 on 10 ng/ml fibronectin-coated (Sigma, UK) T75 tissue culture flasks containing 20 ml of DMEM supplemented with 10% FBS, 1% L-glutamine, 1% PSA (Penicillin-Streptomycin-Amphotericin B) and 1% NEAA that comprised the supplemented culture media and incubated at 37^0C and 5% CO_2. Each T75 flask containing a 10 ml fibronectin coating solution of 10 ng/ml in PBS was incubated for 2 hours at room temperature (RT). Before using the T75 flasks for BMA seeding, fibronectin solution was discarded and the culture media was added. A continuous culture of the whole bone marrow cells containing non-adherent (mononuclear cells) and adherent cells (MSC) was maintained for three weeks in a

humidified incubator at 37^0C and 5% CO_2. Following a 7 day-culture half of media was removed and replaced with antibiotic-free fresh DMEM culture medium as described above. Cell culture continued and on week 2, whole media was discarded, cells were washed once with PBS and supplemented fresh media was added (20 ml/T75 flask). Finally, by week 3 a MSC-colony was formed (also known as CFU-F, colony forming unit- fibroblast). The adherent hMSC population was harvested with trypsin as described next and subsequently passaged for expansion. Before expansion, hMSC were seeded on 24-well plates for their characterisation and immunophenotyping which is described below in the fluorescent immunocytochemistry section 2.7.1.

Table 2.3: Human BMA donor details.

No	BMA label	Donor description	Supplier
1	BMA-8	Human Bone Marrow, Male, Age-35 years	Lonza, USA
2	BMA-11	Human Bone Marrow, Female, Age-26 years	Lonza, USA

Trypsinization for subculture:

During trypsinization process a confluent T75 flask of cells was washed once with 10 ml of PBS (Phosphate-buffered saline) pre-equilibrated at 37^0C and 5 ml of 0.05% trypsin/EDTA solution was added to the cells and incubated at RT for 3-5 minutes. The cells were checked under the microscope and once they looked rounded and detached from each other and the flask's adherence surface the same volume (5 ml) of supplemented media was added to neutralise the action of trypsin. Cell suspension was centrifuged at 1200 rpm (conversion of rpm to g_{av} is shown in Section 2.3.4) for 3 minutes to obtain a cell pellet. The supernatant was

discarded and the cell pellet was resuspended in fresh supplemented media for passage. Media was replaced twice in a week during the whole period of culture.

hMSC expansion:

hMSC were harvested by trypsinisation as described above, expanded in T75 tissue culture flasks and subcultured up to passage 3 by splitting at 1:5 to 1:10 ratios. The DMEM media used for cell culture was supplemented with 10% FBS, 1% L-glutamine, 1% PSA (Penicillin-Streptomycin-Amphotericin B) and 1% NEAA that comprised the supplemented culture media and incubated at 37^0C and 5% CO_2. Passage 1-3 hMSC were utilised for all experimental work.

hMSC cryopreservation:

After isolation from BMA, a large number of hMSC was cryopreserved at Passage-1 for future use. Confluent T75 flasks of hMSC were harvested by trypsinisation and cells stored in the liquid nitrogen at -196^0C in cryovials in freeze-media comprised of 10% DMSO (Dimethyl sulfoxide) in FBS (Foetal bovine serum). The cell content of one confluent T75 flask was stored in each cryovial in 1 ml of freeze media.

2.2.2 hESC culture

hESC line SHEF-1 cells used in this study were obtained from the UK Stem Cell Bank after donation by the University of Sheffield, UK and with accompanying project approval from the Steering Group. hESC were cultured using a freeder-free culture system able to maintain undifferentiated hESCs for at least 130 population doublings (Xu *et al.*, 2001). hESCs were

cultured on matrigel coated T25 tissue culture flasks and their conditioned culture media was prepared using mouse embryonic fibroblasts (MEFs) following the protocol described by Xu et al. 2001.

hESC conditioned media (CM) preparation:

MEFs that were used to condition hESC conditioned media (CM) were isolated from 12.5-13.5 days gravid uteri of black CB1 hybrid mice at small animal facilities, Keele University and were provided by the Stem Cell Group, ISTM, Keele University. hESC CM was composed of KO (Knock out)-DMEM (Gibco, Invitrogen) media supplemented with 20% KO-SR (KO-Serum replacement) (Gibco, Invitrogen), 1% L-glutamine, 1% NEAA, 4ng/ml human bFGF (Basic fibroblast growth factor) and 0.1mM β-mercaptoethanol. hESCs CM was added and cultured/conditioned overnight on semi-confluent MEFs and then collected and further supplemented with 4ng/ml of bFGF and sterile filtered with 0.20 μm porous Millipore filtration unit (Millipore) before use.

Matrigel coating:

Cell culture surfaces of T25 tissue culture flasks were coated with 4 ml /T25 of cold matrigel solution diluted 1:100 in cold KO-DMEM and incubated at RT for 2 hours. Before use, matrigel containing flasks were incubated at 37^0C for 30 minutes in an incubator, then matrigel solution was discarded and hESC culture media was added. Prepared matrigel coated culture flasks were stored at 4^0C for later use.

hESC culture:

A SHEF-1 cryovial (P-28) was taken from liquid nitrogen, equilibrated in a water bath at 37^0C and cells quickly thawed and stored in 10% DMSO in KO-SR solution. Then thawed cells were transferred into a matrigel coated T25 tissue culture flask containing 6 ml CM pre-equilibrated 37^0C and were incubated at 37^0C and 5% CO_2 in the humidified incubator. Media was replaced daily by 6 ml fresh hESC CM. When hESC became 70-80% confluent were harvested by trypsinisation and passaged into 2 to 4 T25 flasks at a split ratio of 1:2 to 1:4. For trypsinisation, cells were washed with PBS and 2 ml of trypsin/EDTA solution was added and incubated for 1-3 minutes at RT. The cells were dislodged by tapping the flask and same volume of hESC culture media was added to the T25. The solution was centrifuged for 3 minutes at 1200 rpm (conversion of rpm to g_{av} is shown in Section 2.3.4) to obtain a cell pellet. Cell pellet was then resuspended in 10 ml of hESC CM and passaged as above split ratio. Cells were passaged every 3 to 7 days.

hESC cryopreservation:

When T25 flasks of hESC were 80-90% confluent they were harvested by trypsinisation and stored in the liquid nitrogen using 10% DMSO in KO-SR freeze media. The cell content of one confluent T25 flask was stored in each cryovial in 1 ml of freeze media.

2.2.3 Cell lines culture

Cell culture for human NCI-H292 and MG-63 as well as mouse NIH-3T3 cell lines was performed according to the suppliers' instructions.

2.2.3.1 MG-63 and NIH-3T3 cell culture

MG-63 and NIH-3T3 cells were stored in the liquid nitrogen at -190^{0}C in cryovials in freeze media comprised of 10% DMSO in FBS. Cells in cryovials were quickly thawed in a water bath at 37^{0}C and resuspended in 10-15 ml of complete culture media that composed of DMEM supplemented with 10% FCS, 1% L-glutamine and 1% PSA.

MG-63 and NIH-3T3 cell suspensions were then passaged into their correspondingly labelled T75 tissue culture flasks and incubated in the humidified incubator at 37^{0}C and 5% CO_2. Their media was replaced 24 hours later with 10-15 ml of fresh complete culture media (as described above). Both cell lines became confluent within 3-4 days. MG-63 and NIH-3T3 cells were subcultured and cryopreserved as described previously (section 2.2.1).

2.2.3.2 NCI-H292 cell culture

NCI-H292 cells were stored in the liquid nitrogen at -190^{0}C in cryovials in freeze media comprised of 10% DMSO in FBS. Cells in cryovials were quickly thawed in a water bath at 37^{0}C and resuspended in 10-15 ml of complete culture media that composed of RPMI 1640 supplemented with 10% FBS, 1% L-glutamine and 1% PSA.

NCI-H292 cell suspensions were then passaged into T75 tissue culture flasks and incubated in the humidified incubator at 37^{0}C and 5% CO_2. Their media was replaced 24 hours later with 10-15 ml of fresh complete culture media (as described above). NCI-H292 cells became confluent within 3-4 days. NCI-H292 cells were subcultured and cryopreserved as described previously (section 2.2.1).

2.3 *In vitro* nanomagnetic gene transfection with the magnefect-nano system

2.3.1 The nanomagnetic gene transfection system components

In this study, for the nanomagnetic gene transfection experiments the novel magnefect-nano system (nanoTherics Ltd, UK) was used to increase the MNP:DNA complexes sedimentation on the cell culture surface, stimulate endocytosis, andpromote further transfection of the complexes. The basic components of the system included the magnefect-nano oscillating array (nanoTherics Ltd, UK), mounted onto a computer-controlled stepper motor to control the lateral sliding/oscillation of the magnet array (nanoTherics Ltd., UK), and the cell culture plates positioned directly above the magnet array holder, with cell culture surface being at 3 mm distance above the magnet array. Following addition of the MNP:DNA complexes into the culture, the cell culture plate and magnefect-nano oscillating system were transferred into an incubator for the duration of transfection (Figure 2.1).

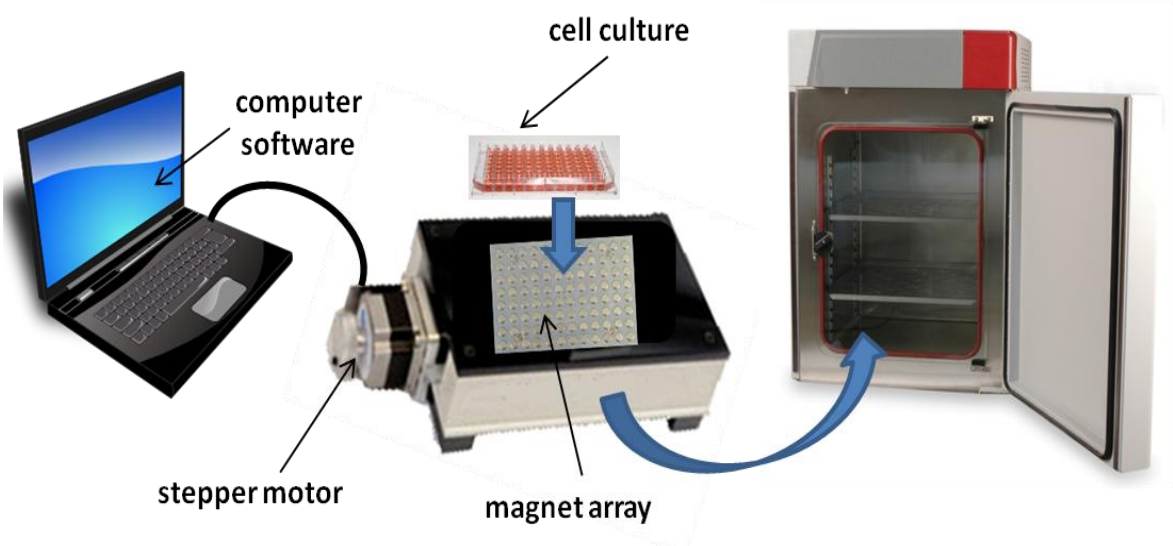


Figure 2.1 The magnefect-nano gene transfection system. The oscillating magnet array of the magnefect-nano system configured for multi-well cell culture plates was mounted to a computer-controlled stepper motor that was interfaced to computer software to control the duration, oscillation frequency and amplitude. For the duration of transfection experiments cell culture and stepper motor were incubated at 37^0C and 5% CO_2 to maintain optimal cell culture conditions.

2.3.2 The magnet arrays

Magnet arrays used for both static and oscillating transfections were configured for 96-well and 24-well tissue culture plate formats. (nanoTherics Ltd., UK), The magnet arrays were constructed arranging cylindrical stacks of rare earth magnet, an alloy of neodymium, iron and boron (NdFeB), with 6 mm and 15 mm diameter correspondingly on non-magnetic aluminium templates (Figure 2.2). Magnetic fields were mapped using the magnetic field scanner, Magtronics MagScan500 (Redcliffe Magtronics, UK).

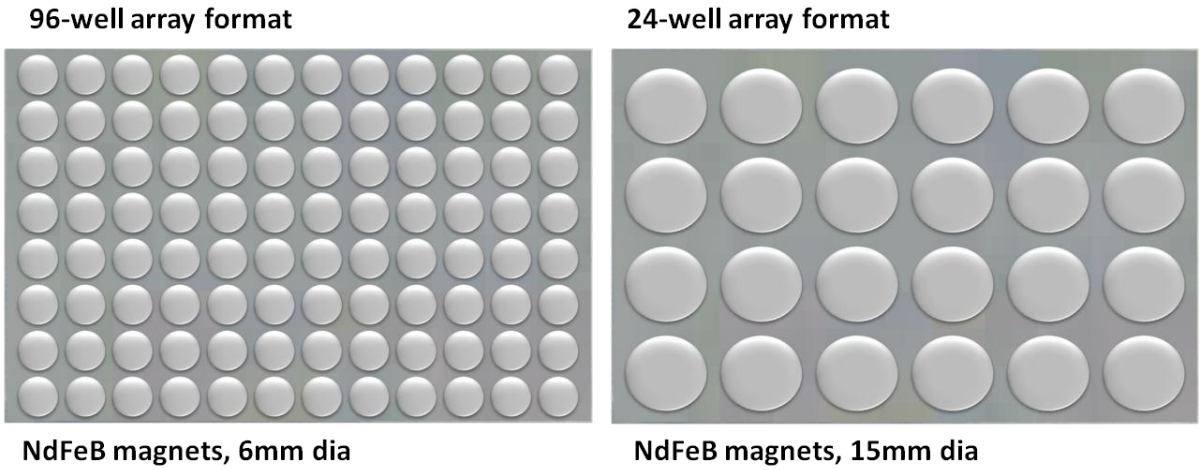


Figure 2.2: Schematic representation of 96-well and 24-well plate formats of the magnet arrays. Cylindrical stacks of NdFeB magnets were used to assemble 96-well (left) and 24-well (right) magnet array format used for static and oscillating transfections.

2.3.2.1 The 96-well magnet array assembly

As described above (Section 2.3.2) the magnet arrays were constructed arranging cylindrical stacks of NdFeB magnet discs on non-magnetic aluminium templates. Here, a detailed description of the 96-well magnet array assembly was provided (Figure 2.3), showing that each cylindrical well at 2 mm above the bottom of the well had a narrowing point, above and below which attracted magnet discs (6 mm diameter, 2 mm height) of opposing magnetic fields were positioned in order to secure the magnets' position into the array. Then, an additional magnet disc (6 mm diameter, 2 mm height) was placed into the array on top of the previously inserted, to reach the top of the cylindrical well. Similarly, all wells of the non-magnetic aluminium template were filled with the magnet discs and the 96-well magnet array was assembled.

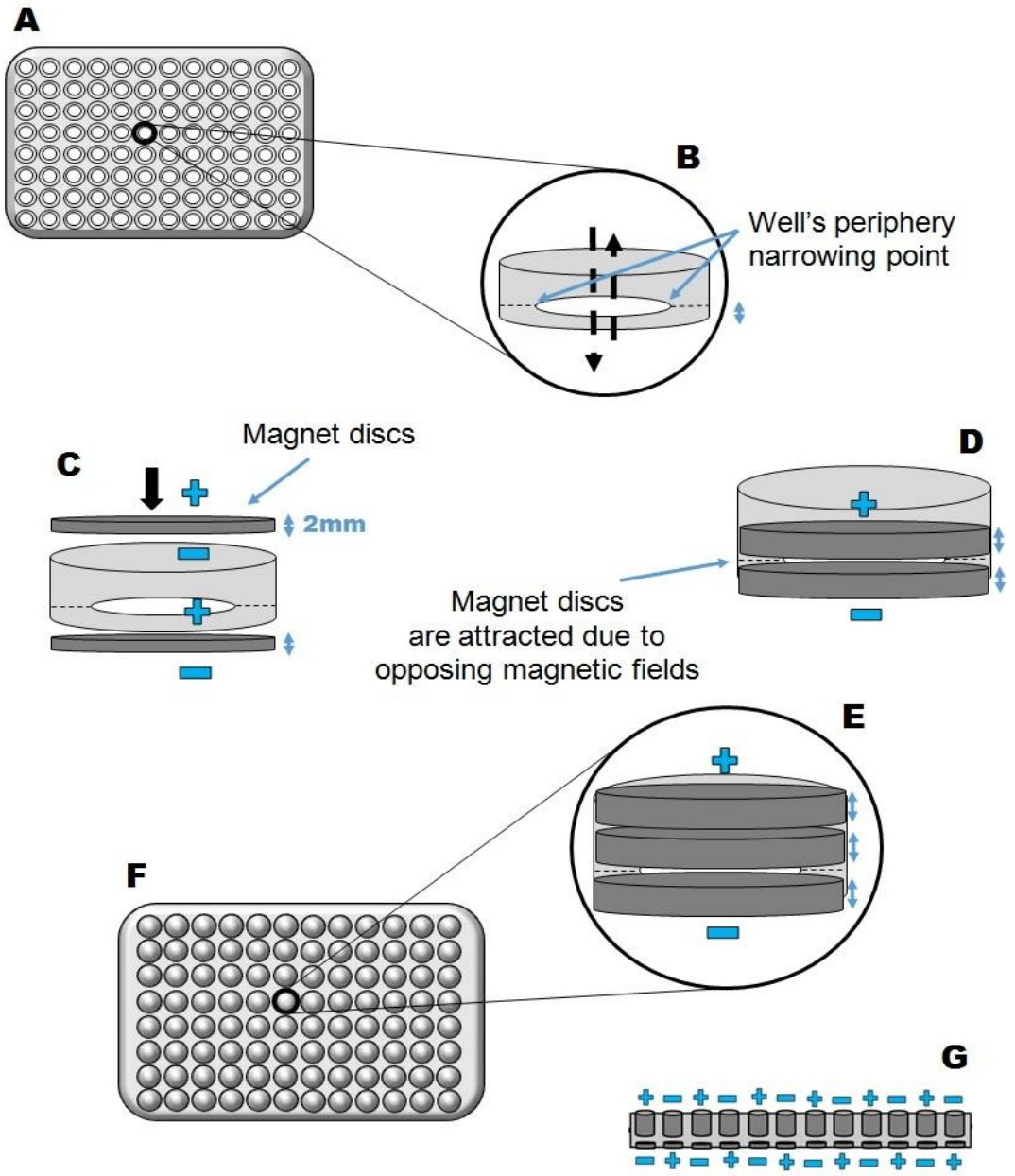


Figure 2.3: Schematic representation of the 96-well magnet array assembly. (A) Top view of the nanoTherics 96-well aluminum plate, empty of magnets. Close up side views of: (B) a cylindrical well the bottom of which is open and the well periphery narrows at 2 mm above the bottom of the well; (C) two NdFeB magnet discs (2mm diameter) are positioned above and below the narrowing point; (D) magnet discs with opposing magnetic fields attracting each other to secure the magnet discs' position; (E) an additional magnet disc

(2mm diameter) positioned on top and attracted by the magnet disc below. (F-G) Top and side view of the fully assembled nanoTherics 96-well magnet array.

2.3.2.2 The magnet height rearrangement of the 96-well magnet array

For the purpose of evaluating the magnetic field requirements for transfection and the resulting effect in transfection efficiency (Section 3.1), the 96-well magnet array format was rearranged in order to vary the distance between the magnet array and cell culture surface (bottom of the culture plate). In particular, the magnet height per column of the magnet array was adjusted in such way that in column 1, magnet discs were positioned directly beneath the cell culture surface of the 96-well plate at the closest of 3 mm. Following on, in columns 2, 3, 4 and 5 the magnet discs were rearranged so the distance from the cell culture surface was increased at 4 mm, 5 mm, 6 mm and 7 mm correspondingly, increasing the distance between the magnet and cell culture surface by 1 mm at each time. Following on from the description of the basic 96-well magnet array assembly (Figure 2.3), the magnet height rearrangements were achieved by using the cylindrical NdFeB magnet discs (6 mm diameter) with a variety of heights (1 mm, 2 mm and 3 mm) (Figure 2.4). The rearranged magnet array used for both static and oscillating transfections was mapped for its magnetic field strength using the magnetic field scanner, Magtronics MagScan500 (Redcliffe Magtronics, UK).

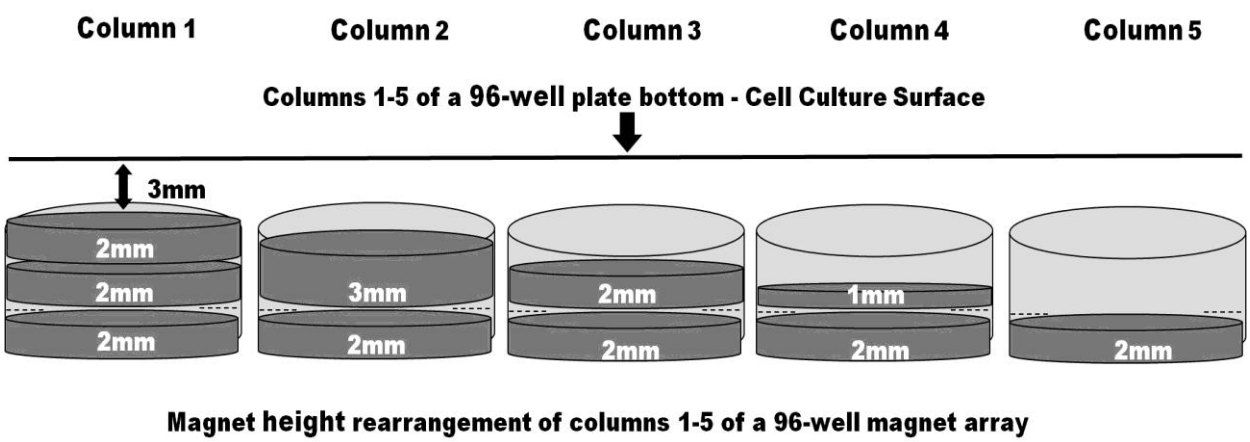


Figure 2.4: Schematic representation of the magnet height rearrangement of the 96-well magnet array. For column 1, magnet discs are positioned directly beneath the cell culture surface of the 96-well plate, at the closest of 3 mm. For columns 2, 3, 4 and 5, magnet discs are rearranged increasing the distance between the magnet and cell culture surface by 1 mm at a time, and therefore keeping the magnets away from the cell culture surface at 4 mm, 5 mm, 6 mm and 7 mm correspondingly.

2.3.3 The magnetic nanoparticles and plasmid DNA

2.3.3.1 The choice of MNPs for transfection

According to the manufacturers, the commercially available SPIONs nTMag (nanoTherics Ltd, UK) and PolyMag (OzBiosciences, France) (0.1mg/ml) used in transfection experiments, have magnetite cores with proprietary multi-layer PEI derivative dispersed in ddH_2O aqueous solution. Also, they have a diameter of approximately 100 nm and are biocompatible and biodegradable when used according to manufacturer's protocol. In addition, both SPIONs have been provided with accompanying quality control information that include in case of nTMag the pH value of suspension (7.0), particle size distribution (1.7) and zetapotential (+23.87mV), and in case of PolyMag the particle size distribution (1.5 to 2.0) and zetapotential (+25 mV to +28 mV). Their appropriate storage conditions were between 4 ˚C to 8 ˚C. The technical characteristics of nTMag and PolyMag as provided by their manufacturers are listed below in the Table 2.4.

Table 2.4: Technical characteristics for commercially available nTMag and PolyMag SPIONs

	SPIONs	
Product name	nTMag	PolyMag
Description	Aqueous dispersion in ddH_2O	
Concentration	0.1mg/ml	

Core	Magnetite	
Type of magnetization	Superparamagnetic	
Coating/ matrix	PEI derivative, multi-layer	
Size (diameter)	100 nm	
Autoclaved	Yes	Unknown
pH value of suspension	7.0	Unknown
Particle size distribution	1.7	1.5 to 2.0
Zeta-potential	+23.87mV	+25 mV to +28 mV
Storage	4 - 8 ˚C	

2.3.3.2 The plasmid DNA as a reporter

The 4.7 kb eukaryotic expression plasmid pEGFP-N1 (Figure 2.5) (Clontech, California, USA) containing a cytomegalovirus (CMV) immediate early promoter/enhancer and gene expressing enhanced Green Fluorescent Protein (EGFP) was used as reporter in this study. The endotoxin free plasmid DNA, purified using EndoFree Plasmid Purification kit (Qiagen, UK) and maintained at -80 ˚C in endonuclease-free water (Sigma, UK), was kindly provided by nanoTherics Ltd. The working concentration of pEGFP-N1 plasmid used for all transfection experiments was 0.2mg/ml.

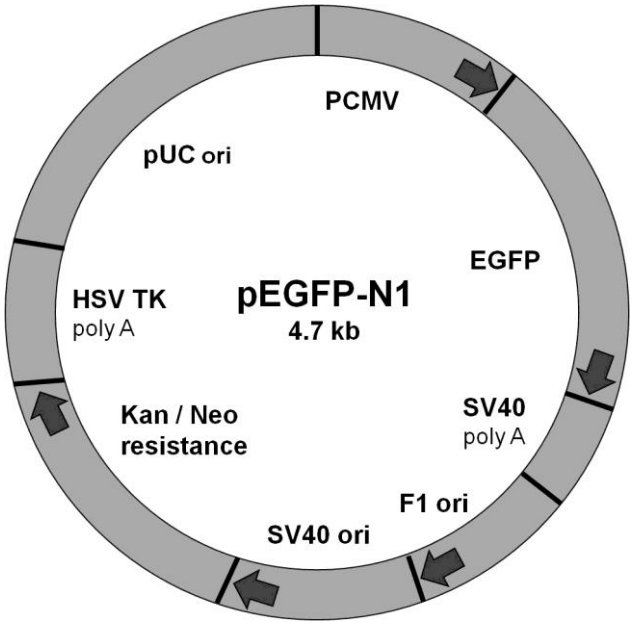


Figure 2.5: Schematic representation of plasmid map of pEGFP-N1 vector. pEGFP-N1 encodes a red-shifted variant of wild type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells, with excitation maximum at 488 nm and emission maximum at 507 nm. EGFP coding sequence is located before the PCMV that represents the cytomegalovirus immediate early promoter responsible for driving expression in mammalian cells. Sequences flanking EGFP have been converted to a Kozak (Kozak consensus translation initiation site) to further increase the translation efficiency in eukaryotic cells. SV40 polyadenylation (poly A) signals protect the mRNA from degradation by exonucleases. F1 ori is the origin of replication for single-stranded DNA production. SV40 ori is the simian virus 40 origin for replication in mammalian cells. Kanamycin/Neomycin resistance and polyadenylation signals from Herpes simplex virus thymidine kinase (HSV TK poly A), allow stably transfected eukaryotic cells to be selected. pUC ori is the origin of replication for propagation of Escherichia coli (E. coli) (Adapted from Yang *et al.*, 1996 and www.clontech.com).

2.3.4 Investigation of MNP:DNA binding

In order to investigate whether the SPIONs to be used for transfection experiments were able to bind pEGFP-N1 containing the gene encoding for enhanced green fluorescent protein (EGFP), MNP:DNA binding curves were performed in DMEM media using spectrophotometry.

A range of different MNP volumes with a concentration of 0.1mg/ml and DMEM media were mixed with 30 μl fixed volume of pEGFP-N1 plasmid (0.2mg/ml) to add up to 60 μl in total which was the total spectrophotometry cuvette sample volume, as shown in Table 2.5. The proportion of DNA that remained unbound (i.e. still in solution after centrifugation) was then determined. A sample containing only plasmid DNA and DMEM but no MNP was assayed as a control. Following mixing of the particles and DNA at RT for 15 min, samples were centrifuged at 14,000 rpm (conversion of rpm to g_{av} is shown below) for 5 min to induce sedimentation of the MNP and/or MNP:DNA complexes. The absorbance of the supernatant containing the unbound DNA was measured for each sample at 260 nm and compared with the DMEM blank sample. In order to determine the proportion of unbound (free) plasmid DNA, spectrophotometry absorbance readings were expressed as percentages of the absorbance of the DNA-only (blank) control and results were plotted in a curve.

Table 2.5: Preparation of spectrophotometry cuvette samples for nTMag and PolyMag MNPs with DNA for binding curves.

nTMag/ PolyMag (µl) original solution	nTMag/ PolyMag (µl) 1:10 dilution/ original solution.	DNA (µl)	DMEM (µl)	Total Volume (µl) Cuvette sample
0	0	30	30	60
0,1	1	30	29	60
0,25	2,5	30	27,5	60
0,5	5	30	25	60
0,75	7,5	30	22,5	60
1	10	30	20	60

Conversion between rpm to g_{av}

The centrifugation conditions for experiments are represented in rpm (revolutions per minute). Additionally, the conversion between rpm to g_{av} (g), i.e. relative centrifugal force (RCF) expressed in units of gravity, is shown next.

Revolutions per minute refer to the acceleration applied to the samples during centrifugation. When rpm unit is used the force of acceleration varies with the radius of the instrument, as the bigger the radius the higher the acceleration. On the contrary, RCF (g) is the same across all instruments. When the value of the radius of the instrument is known, the force of acceleration can be calculated using the formula below:

$$\text{RCF (or g)} = (1.118 \times 10^{-5}) \times \text{rpm}^2 \times \text{r}$$

RCF represents the Relative Centrifugal Force, rpm represents the rotational speed in revolutions per minute, and r is radius of the instrument's rotor in centimeters.

2.3.5 MNP:DNA complexes dose response optimization

In order to test a wide range of MNP volumes complexed with different DNA concentrations and to facilitate optimization of transfection efficiency in different cell types, a MNP:DNA dose response experiment was designed to allow for multiple rations to be tested simultaneously (Figure 2.6). The experiment was designed in a 96-well plate format accommodating duplicate samples of 36 different MNP:DNA ratios. Following this experiment, and observing the expression of GFP under the microscope, the most appropriate MNP:DNA transfection ratio for each cell type we selected.

2.3.5.1 The MNP:DNA dose response protocol

Step 1: Six different DNA concentrations (1.2 μg, 2.0 μg, 3.0 μg, 4.0 μg, 5.0 μg, 6.0 μg) were mixed with 200 μl serum free (SF) media contained in six tubes labeled correspondingly. In parallel, in a 48-well plate 2 μl of MNP were pipetted into wells A1 to A6. The six DNA solutions were added to the six wells of the 48-well plate correspondingly and incubated at RT for 15 min (Step 1, Figure 2.6).

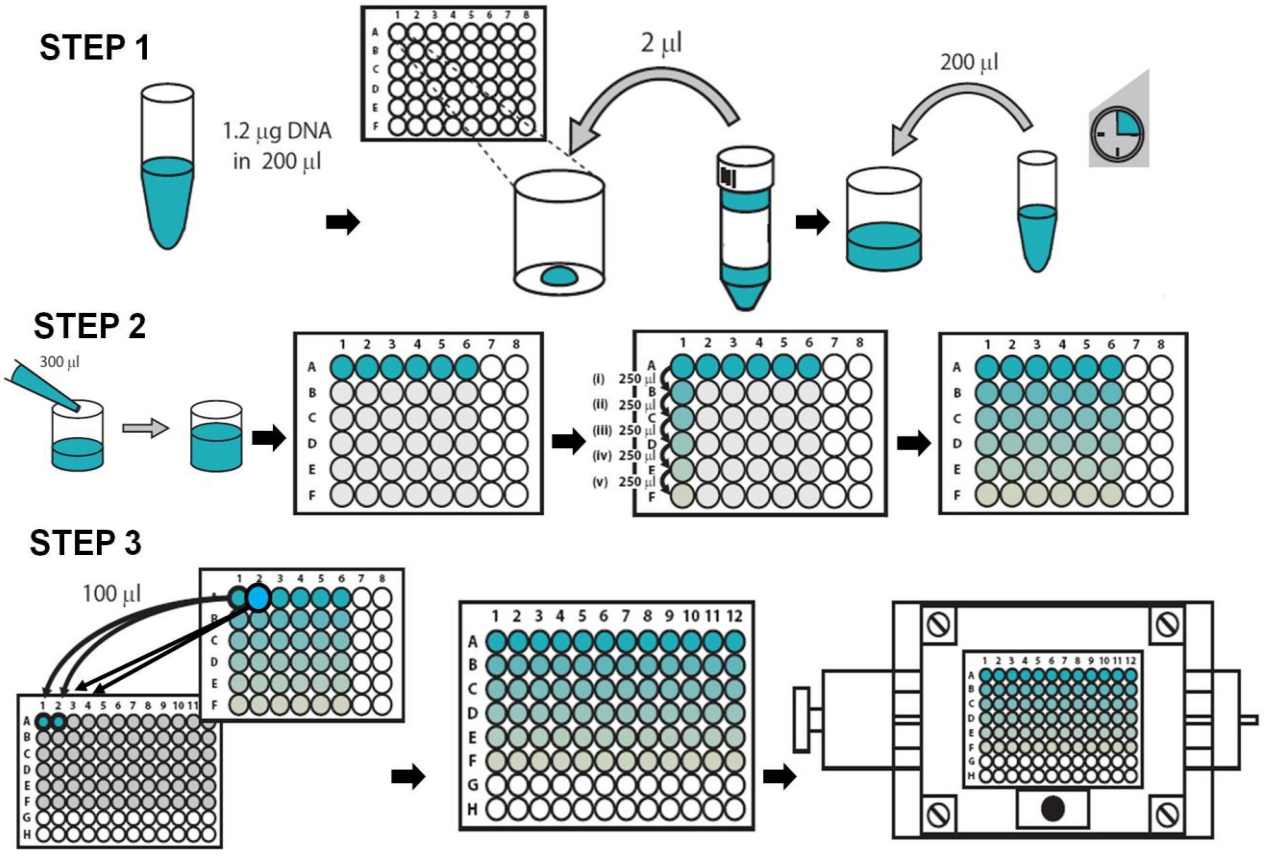
Step 2: Continuing at the 48-well plate, MNP:DNA complexes were diluted to the working concentration by adding 300 μl of supplemented media into all six wells (A1-A6). 250 μl of supplemented media was added into all the remaining wells of the 48-wel plate in columns 1-6. Serial dilutions were performed transferring 250 μl from well A1 below to well B1, mixing well and continuing from B1 to C1 similarly for the remaining wells of column 1. This step was repeated from all 6 columns (Step 2, Figure 2.6).

Step 3: Following serial dilutions at the 48-well plate, 100 μl (in duplicates) of MNP:DNA complex from well A1 (of the 48-well serial dilutions plate) was transferred into wells A1

and A2 of a 96-well tissue culture plate were cells have been seeded for the experiment 24 hours ago. This step was repeated for the remaining wells in the 48-well plate. Then the 96-well tissue culture plate was placed above the oscillating magnet of the magnefect-nano system and was set for 30 min transfection, at 2 Hz frequency and 0.2 mm amplitude (Step 3, Figure 2.6).

Step 4: Following transfection and 48 hours standard incubation of the tissue culture plates, the cells were observed under the microscope for GFP expression and by the use of the table in illustrated in Step 4 the optimal MNP:DNA ratio was determined (Step 4, Figure 2.6).

Following the determination of the optimal MNP:DNA ratio in the 96-well format, and if the transfection experiment was to performed in 24-well plate format (in the case of hESCs) then the surface area of the wells were taken into account. In particular, the surface area of a 24-well plate well (1.9 cm^2) is six times larger than equivalent for a 96-well plate well (0.32 cm^2). In that case the optimal MNP:DNA ratio obtained by the dose response experiment was scaled up by a factor of 6. The same principle can be applied for the seeding densities in larger tissue culture plates.



STEP 4

MNPs µl/96-well		DNA µg/96-well					
		1+2	3+4	5+6	7+8	9+10	11+12
A	0.400	0.240	0.400	0.600	0.800	1.000	1.200
B	0.200	0.120	0.200	0.300	0.400	0.500	0.600
C	0.100	0.060	0.100	0.150	0.200	0.250	0.300
D	0.050	0.030	0.050	0.075	0.100	0.125	0.150
E	0.025	0.015	0.025	0.038	0.050	0.062	0.075
F	0.013	0.008	0.013	0.019	0.025	0.031	0.038

Figure 2.6: Schematic representation of the experimental steps for MNP:DNA dose response optimization. Step 1 illustrates the MNP:DNA complexes preparation of the 6 solutions containing the highest volume/concentration of MNPs and DNA in a 48-well plate. Step 2 explains the serial dilution steps in the 48-well plate, for the preparation of complexes with gradually lower volumes/concentrations of MNPs and DNA, and in adequate numbers for the transfer and transfection at a 96-well tissue culture plate. Step 3 shows the addition of

previously prepared MNP:DNA complexes into the 96-well tissue culture plate, containing cells seeded 24 hrs before. Finally, Step 4 lists all the available MNP:DNA ratios tested during the optimization, and the corresponding wells of the 96-well plate that were added into (Adapted from nanotherics protocols, www.nanotherics.com).

2.3.6 *In vitro* nanomagnetic gene transfection: The protocol

All primary cells (hMSCs and hESCs) and cells lines (NCI-H292, MG-63 and NIH-3T3) used during the experiments were transfected using the oscillating magnefect-nano system (nanoTherics Ltd., UK) and the standard nanomagnetic transfection protocol as described here. All cell type-specific transfection requirements and/or deviations from the standard transfection protocol are described in each cell type-specific paragraph below (Section 2.3.6.1) and summarized in Table 2.6.

Protocol: Cells were seeded onto 96-well or 24-well tissue culture plates at approximately 70% confluency and incubated at 37^0C and 5.0% CO_2 for a period of 24 hours before transfection, to allow cells to adhere to the bottom of the wells. At the transfection experiment day, 24 hrs after seeding, MNP:DNA complexes were composed of 100 µl (in case of 96-well plate) and 400 µl (in case of 24-well plate) of non-supplemented, serum free (SF) cell type-specific media, pEGFP-N1 DNA from a working concentration of 0.2mg/ml and nTMag and/or PolyMag MNPs from an original MNP concentration of 0.1mg/ml for both MNP types used. Following the addition of MNP:DNA complexes to the cell culture, samples were transferred into an incubator at 37^0C and 5% CO_2, and placed over the magnefect-nano oscillating magnet array for 30 min, at 2 Hz horizontal oscillation frequency

and 200 μm amplitude. At 30 min post transfection, cell culture plates were removed from the magnefect-nano system and transfection complexes were replaced with 100μl (in case of 96-well plate) and 400 μl (in case of 24-well plate) of supplemented cell type-specific medium. All samples in tissue culture plates were transferred back into an incubator for 48 hr before data analysis. In the cases of static field experiments, the cell culture plates were placed directly above the magnet array but the oscillation option was not selected and then transferred into an incubator for the duration of transfection as previously described.

The drive system: For oscillating field experiments with the magnefect-nano system, the cell culture plates were placed directly above the magnet array holder that was mounted onto a computer-controlled stepper motor (nanoTherics Ltd., UK) and interfaced to a computer running a software to control the magnet array's horizontal lateral oscillation (nanoTherics Ltd., UK). The cell culture plate and magnefect-nano oscillating system were transferred into an incubator and remained interfaced to control electronics outside the incubator for the duration of transfection (Figure 2.1). The oscillating field transfection experiments were performed at 2 Hz horizontal oscillation frequency and 200 μm amplitude.

Table 2.6: Cell type-specific transfection parameters for nanomagnetic gene transfection.

Transfection Parameters (differing from standard nanomagnetic transfection protocol 2.3.6)	**Cell Type**						
		NCI-H292	**MG-63**	**NIH-3T3**	**hMSC**		**hESC**
Medium type	**96-well plate format**	RPMI 1640	DMEM	DMEM	DMEM	**24-well plate format**	KO-DMEM
SPIONS:DNA complexes- **nTMag and/or PolyMag: pEGFP-N1**		0.1 µl: 0.1 µg			0.2 µl: 0.3 µg		0.6 µl: 0.6 µg
Transfection volume (µl)		100					400
Seeding density		2×10^4	1×10^4	1×10^4	5×10^3		5×10^4
Replacement volume (post transfection)		100					400
Magnets		NdFeB 96-well array format					NdFeB 24-well array format

2.3.6.1 Cell type-specific transfection requirements

NCI-H292 cell line:

Transfection complexes were composed of 100 µl of SF RPMI 1640 media, 0.1 µg of pEGFP-N1 DNA and 0.1µl nTMag MNPs per 96-well tissue culture plate well. All other experimental conditions were as per protocol (Section 2.3.6) and cell culture conditions (Section 2.2.3.2).

MG-63 and NIH-3T3 cell lines:

Transfection complexes were composed of 100 µl of SF DMEM medium, 0.1 µg pEGFP-N1 DNA and 0.1 µl nTMag and/or PolyMag MNPs per 96-well tissue culture plate well. In the case of MG-63 cell line transfections both nTMag and PolyMag MNPs were used, while only nTMag MNPs were used for NIH-3T3 cell line transfections. All other experimental conditions were as per protocol (Section 2.3.6) and cell culture conditions (Section 2.2.3.1).

hMSCs:

hMSCs used for transfection experiments were ranging from 1-3 population doublings. Transfection complexes were composed of 100 µl of SF DMEM medium, 0.3 µg pEGFP-N1 DNA and 0.2 µl nTMag MNPs per 96-well tissue culture plate well. All other experimental conditions were as per protocol (Section 2.3.6) and cell culture conditions (Section 2.2.1).

hESCs:

hESCs used for transfection experiments were ranging from 46-56 population doublings. Transfection complexes were composed of 400 µl of KO-DMEM medium, 0.6 µg DNA and 0.6 µl nTMag MNPs per 24-well tissue culture plate well. All other experimental conditions were as per protocol (Section 2.3.6) and cell culture conditions (Section 2.2.2).

2.4 *In vitro* lipid-mediated gene transfection

All primary cells (hMSCs and hESCs) and cells lines (NCI-H292, MG-63 and NIH-3T3) used during the experiments were transfected using the best currently available cationic lipids, Lipofectamine 2000 (LF2000) (Invitrogen, UK), for the comparison with nanomagnetic transfection. Lipofectamine and nanomagnetic transfections were performed in parallel with identical complex formation conditions, and all other transfection parameters as summarized in Table 2.6. In this section, the exact compositions of Lipofectamine:DNA complexes for each cell type are summarized in Table 2.7. All cell type-specific Lipofectamine transfection requirements and/or deviations from the standard Lipofectamine transfection protocol are described in each cell type-specific paragraph below (Section 2.4.1)

Protocol: Cells were seeded onto 96-well or 24-well tissue culture plates at approximately 70% confluency and incubated at 37^0C and 5.0% CO_2 for a period of 24 hours before transfection, to allow cells to adhere to the bottom of the wells. At the transfection experiment day, 24 hrs after seeding, LF2000:DNA complexes were composed of non-supplemented, serum free (SF) cell type-specific media, pEGFP-N1 DNA from a working concentration of 0.2mg/ml and LF2000 lipid-based agent. Following the addition of LF2000:DNA complexes to the cell culture, samples were transferred into an incubator at 37^0C and 5% CO_2 for 6 hrs as per manufacturers protocol. At 6 hrs post transfection, transfection complexes were removed from samples and replaced with supplemented cell type-specific medium. All samples in tissue culture plates were transferred back into an incubator for 48 hr before data analysis.

The major point of difference in the protocol execution with lipid-mediated transfection, is the requirement for prolonged Lipofectamine:DNA complexes incubation with the cells (6 hrs). This requirement lies on the different mechanism of Lipofectamine:DNA complex uptake by the cells, and the lack of a magnetic component of the complex and a magnet to induce rapid complex sedimentation.

Table 2.7: Cell type-specific composition of Lipofectamine:DNA complexes for transfection.

Transfection Parameters (As shown in Table 2.6)	**Cell Type**						
		NCI-H292	**MG-63**	**NIH-3T3**	**hMSC**		**hESC**
Lipofectamine:DNA complexes- **LF2000: pEGFP-N1**	**96-well plate format**	0.3 µl: 0.1 µg	0.3 µl: 0.1 µg	0.5 µl: 0.2 µg	0.5 µl: 0.2 µg	**24-well plate format**	1.8 µl: 0.6 µg

2.4.1 Cell type-specific transfection requirements

NCI-H292 cell line:

Transfection complexes were composed of 100 µl of SF RPMI 1640 media, 0.1 µg of pEGFP-N1 DNA and 0.3 µl LF2000 per 96-well tissue culture plate well. All other experimental conditions were as per protocol (Section 2.4) and cell culture conditions (Section 2.2.3.2).

hMSCs and MG-63 and NIH-3T3 cell lines:

Transfection complexes were composed of 100 µl of SF DMEM medium, 0.1 µg pEGFP-N1 DNA and 0.3 µl LF2000 for MG-63 cells, and 100 µl of SF DMEM medium, 0.2 µg pEGFP-N1 DNA and 0.5 µl LF2000 for hMSCs and NIH3T3 cells, per 96-well tissue culture plate well. All other experimental conditions were as per protocol (Section 2.4) and cell culture conditions (Sections 2.2.1 and 2.2.3.1).

hESCs:

Transfection complexes were composed of 400 µl of KO-DMEM medium, 0.6 µg pEGFP-N1 DNA and 1.8 µl LF 2000 per 24-well tissue culture plate well. All other experimental conditions were as per protocol (Section 2.4) and cell culture conditions (Section 2.2.2).

2.5 Electroporation of MG-63 cells

In the MG-63 experiments the magnefect-nano system also was compared with the Amaxa Nucleofector (Lonza, UK) electroporation method. MG-63 cells were maintained as described previously and following manufacturers protocol 2 x 10^6 cells were used for a single Amaxa sample. Following electroporation, cell count was performed to determine cell viability and finally samples were seeded onto 96-well tissue culture plates and transferred back into an incubator for 48hr before analysis.

2.6 Fluorescent microscopy

After transfection experiments with the magnefect-nano system, lipid agents and electroporation and following a standard 48 hr incubation, fluorescent microscopy was performed for all transfected samples to visualise GFP-expressing cells. Phase and fluorescent images of transfected cells were captured using a Nikon Eclipse Ti fluorescent microscope.

2.7 Fluorescent Immunocytochemistry

Fixation protocol:

Samples were fixed with 4% PFA (Fisher Scientific, UK) for 30 minutes, washed with PBS once and permeabilized with 0.5% Triton-X 100 (Sigma, UK) for 5 minutes at RT.

Blocking protocol:

Samples were then washed with PBS twice and blocked with 3% BSA for 1 hour at RT. After blocking, samples were washed with PBS once and treated with primary antibodies (Table 2.8) in 0.1% BSA in PBS buffer for 24 hours at 4^0C. The next day samples were washed with PBS twice and secondary antibodies were added and incubated for 2 hours in the dark at RT. For secondary antibody control, samples were treated with secondary antibody (IgG) only. Samples were washed with PBS twice and were immersed in Phalloidin solution at 250 ug/ml PBS for 30 minutes in the dark at RT for actin filament stain. Then samples were washed with PBS once and immersed in DAPI solution (at 1:500) in PBS, for nuclear counter

staining, for 10 minutes in the dark at RT. Samples were then washed three times with PBS and examined under a fluorescent microscope.

2.7.1 Characterisation of hMSCs

5 x 10^3 hMSC (P-1) were seeded on each well of a 96-well plate and grown to 80-90% confluency for immunophenotyping. Cells were fixed with 4% PFA, permeabilised with 0.5% Triton-X 100 and blocked with 3% BSA as described above (Section 2.7). Fixed hMSC cells were characterised using the human MSC characterisation kit containing anti-human mouse anti-CD44, anti-CD90, anti-CD146, anti-CD14, anti-CD19 and anti-STRO-1 primary antibodies at 1:500 dilution (Millipore). Cells were incubated with primary antibodies at 4^0C overnight. Secondary antibodies were anti-mouse IgG-NL557 for all except STRO-1 and anti-mouse IgM-NL493 for STRO-1 (both at 1:200 dilutions) (NorthernLights, R & D System). DAPI was used for nuclear staining. Images were acquired by a fluorescent microscope Nikon Eclipse Ti.

Table 2.8: List of primary antibodies used for immunocytochemistry.

Antibody	Description	Dilution	Company
CD44	Anti-H-CAM antibody Mouse monoclonal (IgG_{2a})	1:500	Millipore, USA Cat No- CBL154-20UL, Component of Cat No- SCR067
CD90	Anti-THY-1 antibody Mouse monoclonal (IgG_1)	1:500	Millipore, USA Cat No- CBL415-20UL, Component of Cat No- SCR067
CD146	Anti-MCAM antibody Mouse monoclonal (IgG_1)	1:500	Millipore, USA Cat No- MAB16985-20UL, Component of Cat No- SCR067
CD14	Anti-CD14 antibody Mouse monoclonal (IgG_1)	1:500	Millipore, USA Cat No- MAB1219-20UL, Component of Cat No- SCR067
CD19	Anti-CD19 antibody Mouse monoclonal (IgG_1)	1:500	Millipore, USA Cat No- MAB1794-20UL, Component of Cat No- SCR067
STRO-1	Anti-STRO-1 antibody Mouse monoclonal (IgM)	1:500	Millipore, USA Cat No- MAB4315-20UL, Component of Cat No- SCR067

2.8 Fluorescence activated cell sorting (FACS)

In order to quantitatively determine the transfection efficiency following transfection experiments with the magnefect-nano system, LF2000 and Amaxa 48 hr after transfection cells were washed with PBS, trypsinized off their culture plates and resuspended in their corresponding supplemented media. Cell suspension was transferred into FACS tubes that were spun down at 1200 rpm (conversion of rpm to g_{av} is shown in Section 2.3.4) for 5 min. The supernatant was discarded and cell pellets were resuspended in FACS buffer (0.1% BSA/PBS). FACS tubes were transferred in an ice bath until the processing. Samples containing at least 20,000 cells (per FACS sample) were counted using a FACSort flow cytometer (Beckton Dickinson, UK) and data was analysed using the CellQuest software package (BD Biosciences, UK).

2.9 Microscopy cell count of transfected cells

Following transfection at 48 hrs during data analysis, transfection efficiency of the gene delivery methods used was estimated by microscopy counts, for cells expressing GFP where no FACS count was performed. Based on the fluorescence microscopy images obtained, both the number of GFP-expressing cells and the total number of cells were counted, and expressed as a percentage of transfection efficiency by the following calculation.

Transfection Efficiency (%) = (no. of GFP-expressing cells / total no. of cells) x 100

2.10 Reverse transcription-PCR (RT-PCR)

Semi-quantitative RT-PCR analysis was performed to determine the mRNA expression of Oct-4, hTERT, Nanog, AFP, ACTC1, SOX1 and ACTB as the house keeping gene in hESC. The primers were designed using human gene sequences from NCBI Map Viewer and Primer 3. Designed primers were evaluated in NCBI Primer-BLAST to check specificity and customised primer sets were purchased from Invitrogen, USA. Primers are tabulated below (Table 2.9). RT-PCR was performed with a one-step protocol.

Table 2.9 Primer sequences. Forward and reverse primers are listed in 5' to 3' orientation. All primers were obtained from Invitrogen, USA.

Gene	Primers (5'-3')		Annealing temp (^{0}C)	Amplicon size (bp)
POU5F1 (Oct-4)	F	GCAATTTGCCAAGCTCCTGAAGCAG	55	536
	R	CATAGCCTGGGGTACCAAAATGGGG		
TERT (hTERT)	F	GCAGCTCCCATTTCATCAGC	53	343
	R	CAGGATGGTCTTGAAGTCTG		
NANOG (Nanog)	F	GGTGGCAGAAAAACAACTGGC	55	300
	R	TGCAGGACTGCAGAGATTCC		
AFP	F	CAGAAAAATGGCAGCCACAGC	54	400
	R	TGGCAGCATTTCTCCAACAGG		
ACTC1	F	CATCCTGACCCTGAAGTATCCCATC	56	315
	R	CCCTCATAGATGGGGACATTGTGAG		
SOX1	F	CCAGGAGAACCCCAAGAGGC	56	206

	R	CGGCCAGCGAGTACTTGTCC		
ACTB	F	GCCACGGCTGCTTCCAGC	55	505
(β-actin)	R	AGGGTGTAACGCAACTAAGTC		

RNA extraction:

Total RNA was extracted using the QIA RNeasy Mini Spin Column (Qiagen, Germany) according to the manufacturer protocol. For RNA extraction preparation adherent hESC were harvested by trypsinisation, cells washed with PBS once and pellets were formed by centrifugation. Cell pellets were lysed with RLT lysis buffer (350 µl RLT buffer + 3.5 µl β-mercaptoethanol per sample). Cell lysates were transferred into the upper column of the QIA Shredder, centrifuged for 3 minutes at 13,000 rpm (conversion of rpm to g_{av} is shown in Section 2.3.4) then collected in the collection tube and stored at -80^{0}C. For RNA extraction, previously stored cell lysates were thawed on ice and 350 µl of 70% ethanol added. The cell lysate-ethanol solution (700 µl total) was transferred into the upper column of QIA RNeasy Mini Spin Column and centrifuged for 25 seconds at 6000 rpm (conversion of rpm to g_{av} is shown in Section 2.3.4). Flowthrough was discarded and 700 µl of RW1 buffer was added and centrifuged for 25 seconds at same speed. Then flowthrough was discarded again and 500 µl of RPE buffer was added into the upper column and centrifuged for 25 seconds. This step was repeated with 2 minutes of centrifugation at maximum speed. Collection was discarded and the upper column was transferred into a fresh collection tube and further centrifuged for 1 minute at maximum speed. Into this 20 µl of RNAse/DNAse-free dH_2O was added and allowed to stand vertically for 1 minute. Column was centrifuged for 1 minute at

maximum speed and this step was repeated once for maximum yield of RNA. The extracted RNA was collected into the eppendorf. The quality and quantity of RNA yield were determined by measurement of absorption at 260 and 280 nm using NanoDrop ND-1000 spectrophotometer (NanoDrop, USA). The ratio of optical density at 260 and 280nm was >1.8 in all cases.

One-step RT-PCR:

One-step RT-PCR was performed using SuperScript® III One-Step RT-PCR System with Platinum® *Taq* High Fidelity kit (Invitrogen, USA) and amplified in DNA Engine® Thermal Cycler (MJ Research, USA) in a single tube according to the manufacturer protocol. In short, 10 ng RNA of each sample was mixed with 6.25 µL 2X Reaction Mix, 3 µL RNAse-free water, 1 µL of each primer (10 µM) and 0.25 µL of Platinum® *Taq* polymerase enzyme.

The thermal cycling protocol steps comprised an initial reverse transcription at 50^0C for 30 minutes, pre-denaturation at 94^0C for 2 minutes followed by 30 cycles of denaturation at 94^0C for 15 seconds, primer specific annealing temperatures (depending on primers as shown in table 2.9) for 30 seconds and extension at 68^0C for 1 minute followed by a final extension at 64^0C for 5 minutes. RT-PCR products were separated by 2% agarose gel electrophoresis (Fisher Scientific, USA) and visualized by ethidium bromide staining (Sigma, USA) under UV light. Primers for β-actin were used as a loading control. Imaging of the RT-PCR products was performed with Gene Snap software.

Agarose gel protocol:

The RT-PCR products were separated by electrophoresis on 2% agarose gel. For the gel preparation 2 g of agarose powder (Fisher Scientific, USA) was dissolved in 100 ml of 1X TAE (Tris Acetate-EDTA) buffer in the microwave oven until completely dissolved. Then 5 µl of ethidium bromide (10 mg/ml) was added and mixed properly and the hot agarose solution was then immediately poured onto an agarose gel case and a comb was placed to make pockets. After 45 minutes at RT, the gel was formed and loaded with samples. The agarose gel was placed on the electrophoresis machine (Bio-Rad DNA Sub Cell) and covered in 1X TAE buffer. Each sample (RT-PCR product) was mixed with 2 µl of loading buffer. 6 µl of PCR product was then loaded per well. 6 µl of DirectLoad^{TM} wide range DNA marker (Sigma) was loaded into two wells to determine product size after electrophoresis. Electrophoresis was performed for 45 minutes with 100 volts constant current.

2.11 Cell Viability

Following 48 hr incubation post-transfection, samples were assayed with the Cytotox-ONE Homogeneous membrane integrity assay (Promega, UK) for their cell viability as per the manufacturer's protocols. This method was used to assess membrane integrity of the cells as a proxy for viability following transfection by measuring LDH release from cells into the culture medium. In brief, for 96-well plate formats, assay plates were equilibrated at RT. Lysis solution (2 µl) was added to the cells that would serve as positive control (100% cell death). CytoTox-ONE reagent (100 µl) was added in each well, cell culture plate was gently shaken for 30 seconds and incubated at RT for 10 minutes. Stop solution (50 µl) was added to

all wells and plate was shaken for 10 seconds. Fluorescence with an excitation wavelength of 560 nm and an emission wavelength of 590 nm was recorded by a Biotech Synergy plate reader (Biotech, USA).

2.12 Statistical analysis

Data from transfection efficiency and cell viability were analysed for statistical significance using the GraphPad Prism® (version 5.00 for windows) (GraphPad Software, USA) statistical analysis package. Statistical significance of compared data sets were analysed by a one-way ANOVA using a Tukey-Kramer multiple comparison test and a 'p' value less than 0.05 (<0.05) was considered indicative of statistical significance. *In vitro* data are presented as mean ± standard error of mean (SEM), and at least three separate repeats were performed for each experimental design which is referred as 'n' number.

2.13 Study approval

Human tissue research was approved by the Guy Hilton Research Centre, Institute for Science and Technology in Medicine, Keele University, Genetic Modification of Microorganisms safety committee. All research involving hESC was performed within a UK Human Tissue Authority licensed premises.

Chapter 3

Results

(experiments carried out between 11/2008-05/2012)

3.1 Evaluation of the magnetic field requirements for nanomagnetic gene transfection

3.1.1 Objectives of this study

As described in the introduction chapter (section 1.7.1), an applied magnetic field must have a gradient in order to generate a force on the MNP, as in the presence of a homogeneous field the particle will experience no force. In this study, the effects of the distance (and by proxy, magnetic field strength) between the magnet and the cell culture surface on transfection efficiency results were investigated for static and oscillating field transfection in order to: (i) evaluate the magnetic field gradient that could create a threshold above which the magnetic field force rapidly increases MNPs sedimentation accelerating endocytosis of MNPs by the cells, and (ii) investigate whether cellular morphology and viability are affected following transfection and exposure to different magnetic field levels during transfection.

3.1.2 Study design

Previous work by our group showed that protein production increased significantly, showing an up to 10-fold increase, as a result of transfection efficiency in relation to the oscillating magnetic field strength (Fouriki *et al.*, 2010).

In this work we investigated the capacity of nTMag MNPs to bind pEGFP-N1 plasmid DNA that contained the gene encoding for enhanced green fluorescent protein (EGFP), and transfected NCI-H292 cells with nTMag:DNA complexes on a 96-well plate format for 2 hours using static and oscillating magnetic fields. Transfection with the oscillating field (magnefect-nano system) was performed at 2 Hz frequency and 0.2 mm amplitude. In

addition, and in order to determine to optimal distance between the magnet and cell culture surface the 96-well format magnet array was rearranged so that the distance between the magnet array and the cell culture would increase by 1 mm in each column of the magnet array. Furthermore, this experiment would enable us to visualize the transfection efficiency results by fluorescent microscopy and to determine whether the application of nTMag:DNA complexes and static and oscillating magnetic fields had any adverse effects on the morphology and viability of our samples following treatment using Cytotox-ONE cell viability assay.

3.1.3 Results

3.1.3.1 Determination of the magnetic field strength

In order to determine the optimal working distance between the magnet (and by proxy, the magnetic field strength) and cell culture for the improvement of transfection efficiency in NCI-H292 cells the magnet arrays were rearranged as described in chapter 2, sections 2.3.2.1 (Figure 2.3) and 2.3.2.2 (Figure 2.4) in the following format: In column 1 magnet discs were positioned directly beneath the cell surface of the 96-well plate (at 3mm). Following, in columns 2, 3, 4 and 5 the magnet discs were rearranged so the distance between the magnet and the cell surface (bottom of the culture plate) was at 4mm, 5mm, 6mm and 7mm correspondingly and therefore increasing the distance between the magnet and cell surface by 1 mm at a time. The rearranged magnet array used for both static and oscillating transfections was mapped for its magnetic field strength as shown in Figure 3.1. The cylindrical stacks were assembled to align with 96-wel plates produced magnetic fields of up to ~101 millitesla (mT) at the cell surface.

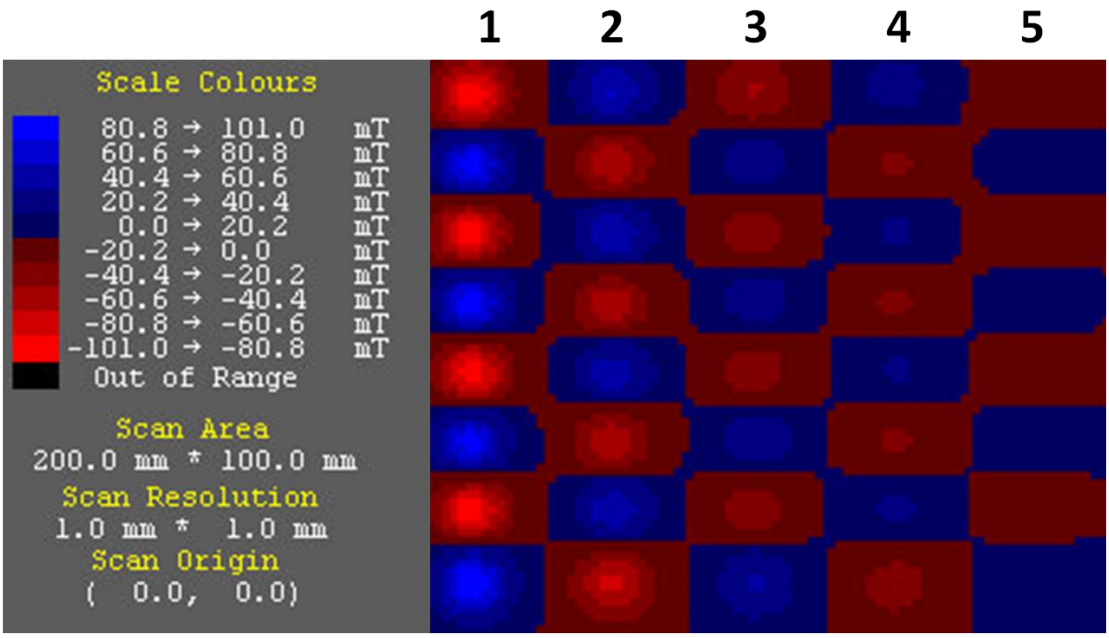


Figure 3.1: Redcliffe MagScan image of a nanoTherics Ltd. Magnet array obtained at 3 mm distance between the scanning probe and the magnet surface. This magnet array has been rearranged for the purposes of the experiment as described earlier, to gradually increase the distance between the magnet array and the cell culture between 3mm to 7mm. At the centre of the wells in column 1 (at 3 mm distance) the highest magnetic field was obtained reaching 101 mT (Image redrawn after Fouriki *et al.*, 2010).

3.1.3.2 MNP:DNA binding and transfection of NCI-H292 cells

As described in chapter 2, section 2.3.4, SPIONs nTMag and pEGFP-N1 were used for the binding curve investigation before transfection.

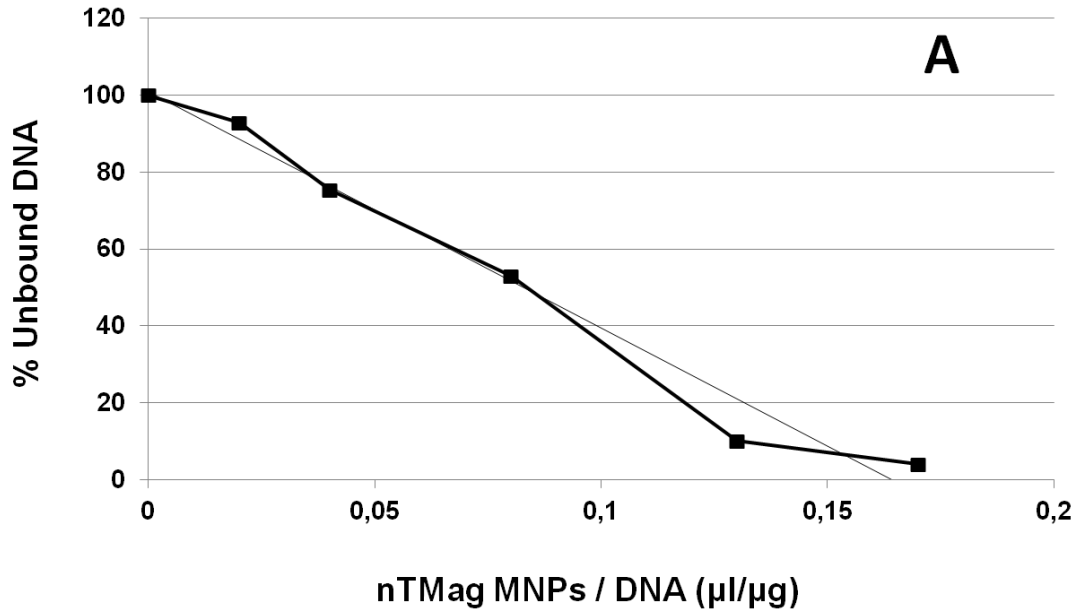


Figure 3.2: Typical DNA binding curve showing the nTMag MNPs: pEGFP-N1 plasmid ratio of binding. Particle saturation levels were reached nearly at 0.1 µl nTMag MNPs and 0.1 µg DNA.

A binding assay demonstrated that nTMag MNPs and pEGFP-N1 DNA were efficiently bound and formed complexes as shown in Figure 3.2. The optimal nTMag MNP:DNA ratios used to form the transfection complexes for NCI-H292 transfection were those that nearly reached particle saturation levels (0.1 µl nTMag MNPs and 0.1 µg DNA).

Once transfection complexes were formed and added to the cell culture, static and oscillating magnets (F = 2 Hz and amplitude = 0.2 mm) were applied beneath using the rearranged magnet array from Figure 3.1 for 2 hours. Following 48 hours of incubation

fluorescent microscopy images demonstrated an increase in GFP-expression levels between the samples transfected with nTMag MNPs exposed to the oscillating field for 2 hours, in comparison to those transfected with nTMag MNPs in the presence of no field or static field. In addition, the fluorescent images show a small but gradual decrease in GFP-expressing cells as the magnetic field strength decreases, and hence when the distance between the magnet array and the cell culture increases (Figure 3.3).

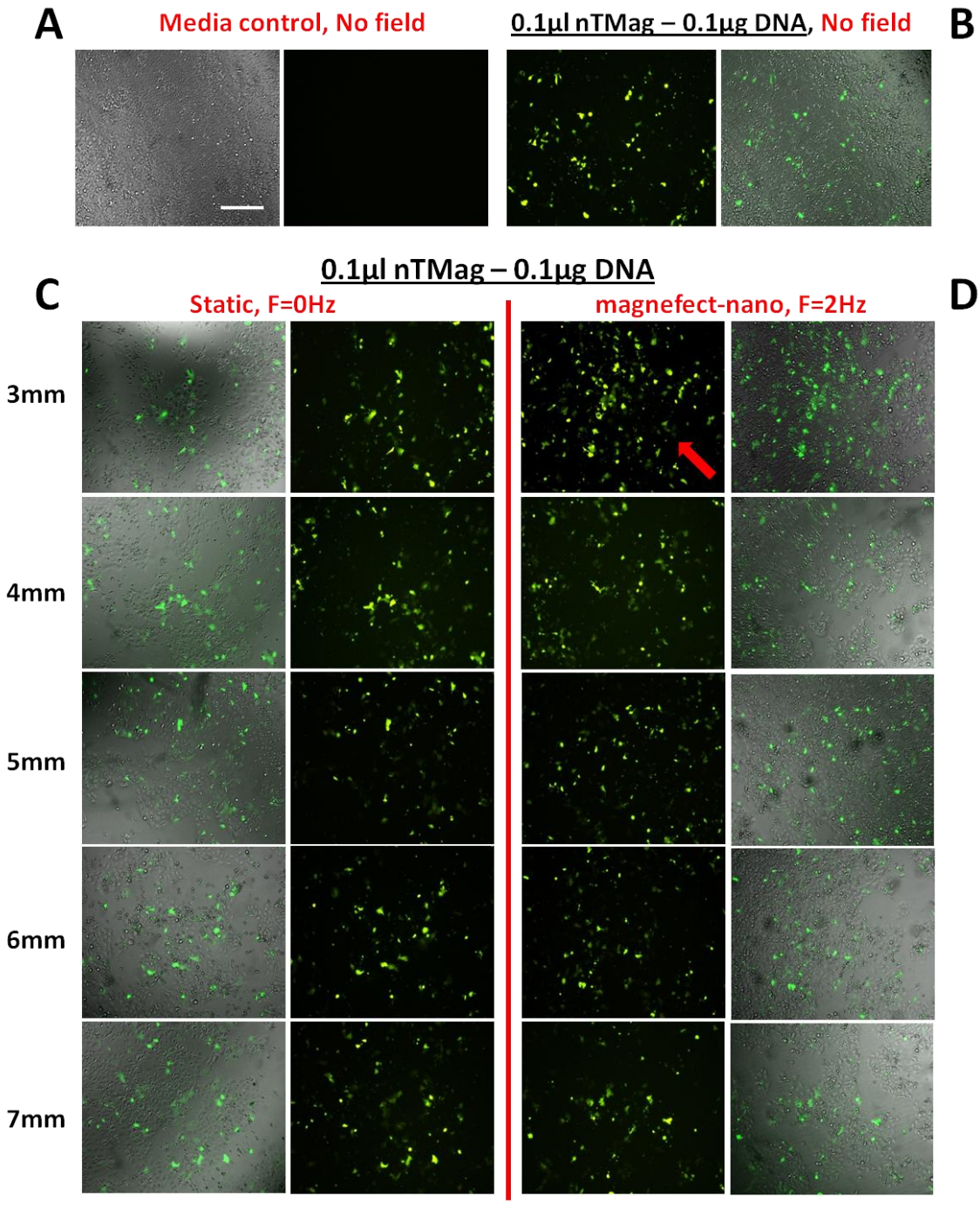


Figure 3.3 Fluorescent microscopy images of NCI-H292 cells expressing GFP following transfection at different magnet array distances from the cell culture surface. (A) Untreated, (B) transfected with 100 nm nTMag MNPs coated with pEGFPN1 plasmid DNA; in the absence of a magnetic field, for 2hr (C), in the presence of a static

field (nanoTherics static array) for 2 hr (D) and an oscillating field (nanoTherics magnefect-nano array at F = 2 Hz and amplitude = 0.2 mm), for 2 hr. Cell seeding density was $2x10^4$/ 96-well, incubation period 48 hr (at 37 ˚C, 5 % CO_2) post transfection and scale bar = 100μm in (A-D).

3.1.3.3 Cell viability studies following transfection of NCI-H292 cells

At 48 hr post transfection, the cell viability and the corresponding toxicity of the nTMag MNPs:DNA complexes for no field, static and oscillating field conditions were tested using a Cytotox-ONE Homogeneous membrane integrity assay that measures LDH release from cells with non-intact cell membranes. From the cell viability/toxicity bar chart it can be seen that cell viability of transfected NCI-H292 cells using nTMag MNPs at no field, static and oscillating magnetic field conditions at 2 hours were similar, and marginally lower relative to untreated controls (statistically insignificant) showing that treatment with nTMag MNPs:DNA complexes and magnetic fields had no significant effect on cell viability (Figure 3.4). In regards to samples transfected at various distances from applied the magnetic cell viability remained unaffected indicating that the magnetic field strength had no adverse effect cell viability, however it might improve transfection efficiency.

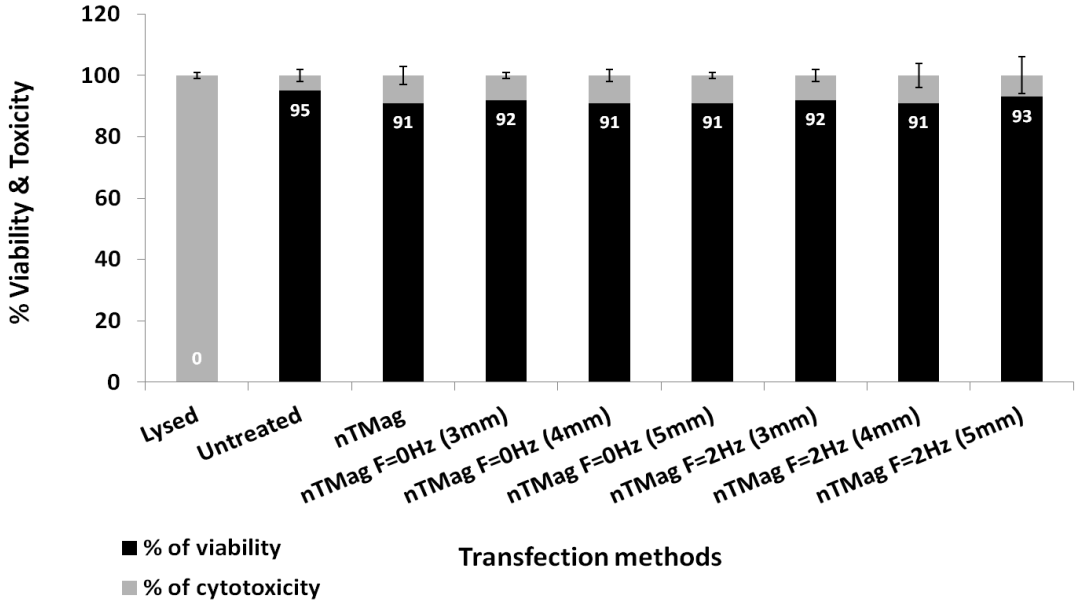


Figure 3.4 Cell viability of transfected NCI-H292 cells. Bar chart showing combined average percentage for viable and non viable cells following treatment with nTMag MNPs:DNA complexes and 'no field', 'static field' (F=0Hz) and 'magnefect-nano' (F=2Hz) transfections at 2hr, at 48hrs post transfection. n=9 for all test samples.

3.2 Efficient Transfection of MG-63 Osteoblasts Using Magnetic Nanoparticles and Oscillating Magnetic Fields

3.2.1 Objectives of the study

In this study the potential of MNPs in transfecting human osteosarcoma fibroblasts (MG-63) was examined as well as the effects of the novel non-viral oscillating nanomagnetic gene transfection system (magnefect-nano™) in enhancing transfection efficiency at shorter transfection times (30 min transfection duration). The nanomagnetic gene transfection method was performed using two different type of superparamagnetic iron oxide MNPs and it was also compared with lipid mediated gene delivery and electroporation techniques.

3.2.2 Study design

MG-63 cells were transfected using MNPs coupled with a GFP-carrying plasmid. The magnefect-nano system was evaluated for transfection efficiency at shorter transfection times and fluorescent microscopy was used to visualize GFP-expressing cells following transfection. The method was compared with lipid mediated gene delivery (cationic lipids) (6 hour duration) and electroporation according to the manufacturers protocol. Furthermore, cell viability studies were used to determine whether the application of the nTMag/ and PolyMag MNPs:DNA complexes and static and oscillating magnetic fields had any potential associated effects on cell viability following treatment using Cytotox-

ONE cell viability assay and compared with the equivalent results from lipid mediated gene delivery and electroporation.

3.2.3 Results

3.2.3.1 Determination of the magnetic field strength

In order to determine the magnetic field strength, the magnet arrays were rearranged as described in chapter 2, sections 2.3.2.1 (Figure 2.3) in the following format: In all columns magnet discs were positioned directly beneath the cell surface of the 96-well plate (at 3mm). The magnet array used for both static and oscillating transfections was mapped for its magnetic field strength as shown in Figure 3.5. The cylindrical stacks were assembled to align with 96-wel plates produced magnetic fields of up to ~101 millitesla (mT) at the cell surface.

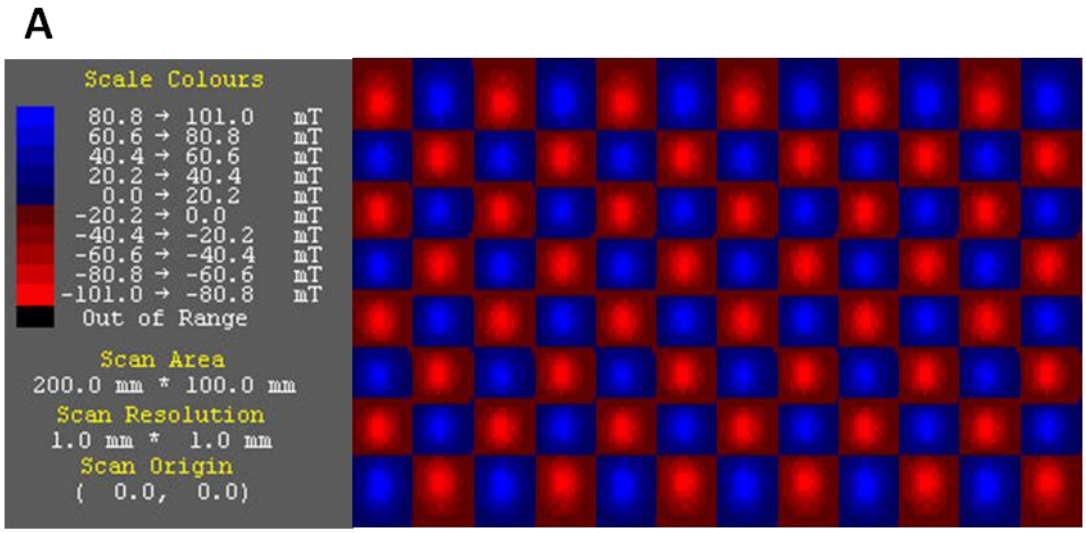


Figure 3.5: Redcliffe MagScan image of scale colours of magnetic field mapping for the nanoTherics Ltd. 96-well format magnet array. Magnet array obtained at 3 mm distance between the scanning probe and the magnet surface. At the centre of each well (at

3 mm distance) the highest magnetic field was obtained reaching 101 mT (Fouriki *et al.*, 2010).

3.2.3.2 MNP:DNA Binding and Transfection of MG-63 cells

As described in chapter 2, section 2.3.4, two types of SPIONs, nTMag and PolyMag, were associated with pEGFP-N1 for the binding curve investigation before transfection. nTMG/plasmid binding curve was described in Figure 3.2. Furthermore, PolyMag/plasmid binding curve is decribed here (Figure 3.6).

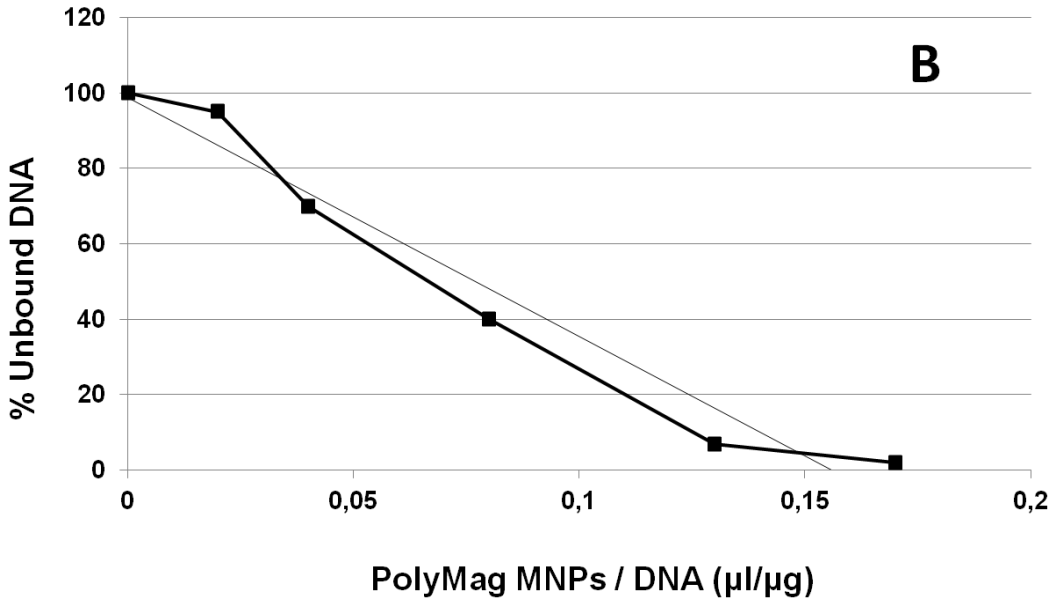


Figure 3.6: Typical DNA binding curve showing the PolyMag MNPs: pEGFP-N1 plasmid ratio of binding. Particle saturation levels were reached nearly at 0.1 µl nTMag MNPs and 0.1 µg DNA.

Binding assays demonstrated that nTMag and PolyMag MNPs could efficiently bind pEGFP-N1 DNA as shown in Figure 3.2 and Figure 3.6 (as described in section 2.3.2). The optimal nTMag/ and PolyMag MNP:DNA ratios used to form the transfection complexes for MG-63 cells transfection were those that nearly reached particle saturation levels for both particles that demonstrated very similar binding curves (0.1 μl MNPs and 0.1 μg DNA).

Once transfection complexes were formed and added to the cell culture, static and oscillating magnet arrays (F = 2 Hz and amplitude = 0.2 mm) were placed beneath the cell culture for 30 min. In parallel, transfection with lipid mediated gene delivery (Lipofectamine 2000, LF2000) and electroporation were performed as described in methods section. Following 48 hours of incubation fluorescence microscope images clearly show the highest GFP-expression levels in the samples exposed to magnetic fields, both static and oscillating (Figure 3.7). Electroporation and lipid-based transfection were not as effective. Some transfection was seen when using the nTMag particles even in the absence of an applied field. For both static and oscillating magnet conditions, nTMag particles appeared to provide more efficient transfection.

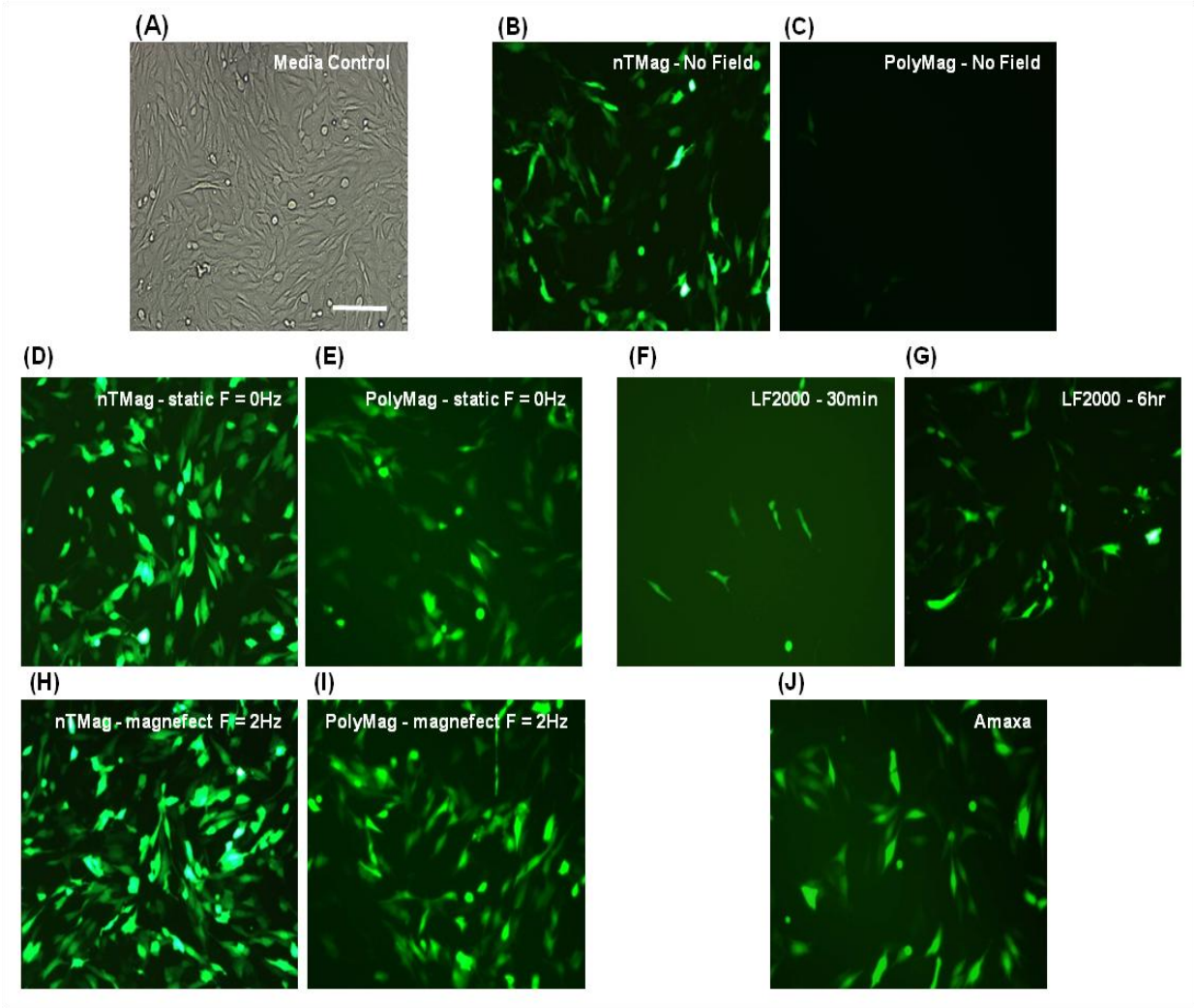


Figure 3.7: Transfection of MG-63 cells. **(A)** Light image of untreated human MG-63 cells. **(B-J)** EGFP expression in MG-63 cells; transfected with 100 nm nTMag and PolyMag MNPs coated with pEGFPN1 plasmid DNA in the absence of a magnetic field, for 30 min **(B-C)**, in the presence of a static field (nanoTherics static array) for 30 min **(D-E)** and an oscillating field (nanoTherics magnefect-nano array at F = 2 Hz and amplitude = 200 μm), for 30 min **(H-I)**. Transfected with Lipofectamine 2000^{TM}, for 30 min **(F)**, for 6 hr **(G)** and electroporated with Amaxa nucleofector system ($5x10^5$ / sample) **(J)**. Incubation period (48 hr, 37 ˚C, 5 % CO_2) post transfection and electroporation were identical for all samples. Cell seeding density was $1x10^4$/well (A-I). Scale bar = 200μm in (A-J). Data

represent at least three independent experiments (N=3). (Image taken from Fouriki et al., 2012).

3.2.3.3 Quantitative Analysis of transfection efficiency (FACS)

Quantitative analysis of transfection efficiency obtained using Fluorescence-Activated Cell Sorting (FACS) provided confirmation of the qualitative results seen via fluorescence microscopy (Figure 3.7). In the bar chart, the effect of the oscillating magnet arrays (F=2 Hz) using both nTMag and PolyMag MNPs at 30 min was compared to "no field" and "static field" (F=0) conditions (at 30 min), as well as lipid-mediated gene transfection and electroporation methods.

Again, some GFP expression (12%) representing the transfection efficiency was observed in the presence of nTMag MNPs:DNA complexes even in the absence of a magnetic field. However, a dramatic increase in GFP-expressing MG-63 cells (49%) was demonstrated when nTMag MNPs:DNA complexes were introduced to a static magnetic field, and an even greater transfection efficiency (53%) was shown when introduced to the oscillating magnetic field (magnefect-nano system, nanoTherics). The increase in transfection efficiency observed between the no field and both the static and oscillating field conditions was found to be statistically significant (***$p<0.001$). In addition, transfection efficiency using both static and oscillating magnetic fields (at 30 min) was shown to significantly outperform Lipofectamine 2000 at both 30 min (7%) and 6 hr (24%) as well as Amaxa Nucleofector (21%) (***$p<0.001$).

However, when PolyMag MNPs:DNA complexes were used to transfect MG-63 cells at both static (24%) and oscillating (20%) field conditions, overall transfection efficiency was significantly lower than the nTMag for the same conditions (***p<0.001) (Figure 3.5).

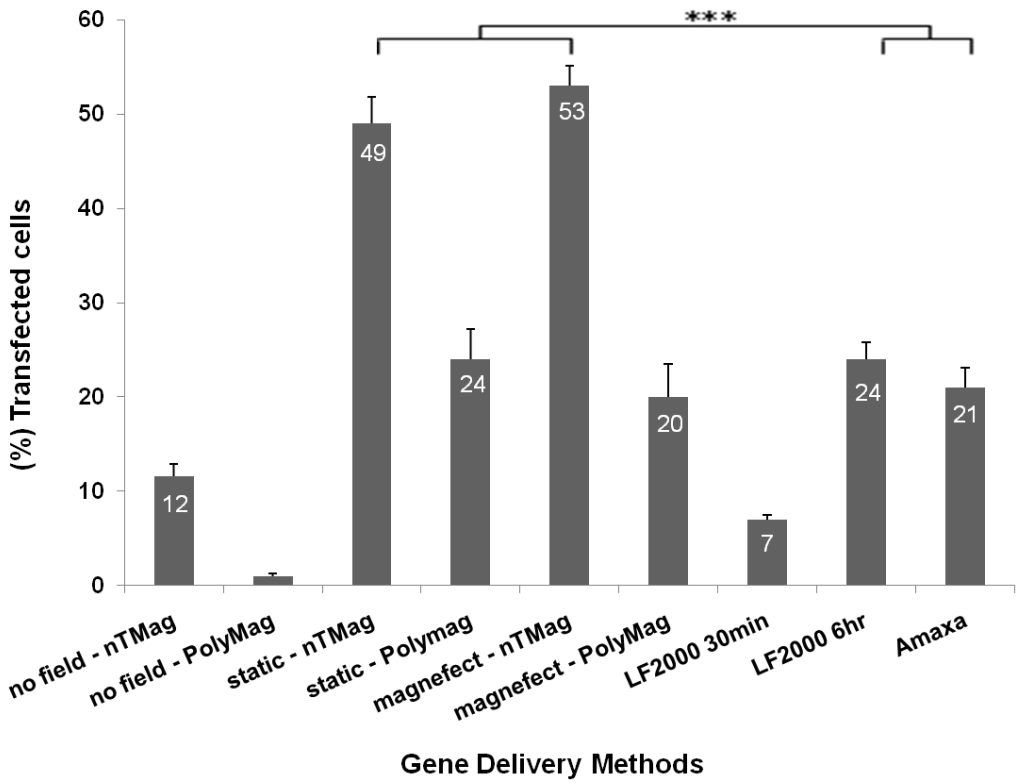


Figure 3.8: Bar chart of quantitative analysis of MG-63 transfection. Average of FACS data from transfection efficiency of GFP-expressing MG-63 cells transfected with nTMag and PolyMag MNPs coated with pEGFPN1 DNA in the absence of a magnetic field (n=9), in the presence of the nanoTherics static magnetic array (n=7) and the nanoTherics magnefect-nano™ oscillating magnetic array (200 µm amplitude, 2 Hz) (n=9) for 30 min, in comparison with lipid-based transfection (LF2000, for 30 min and 6 hr) (n=9) and electroporation (Amaxa) methods (n=8). Data represent the mean ±SD of at least three independent experiments (N=3). During transfection cells were incubated at 37˚C and

5% CO_2. At 30min post transfection the magnets were removed and cells were incubated for 48 hr before analysis. (Image taken from Fouriki *et al.*, 2012)

Following Figure 3.8, summarizing the average of FACS data from transfected MG-63 cells, next figure (Figure 3.9) shows the GFP expression data, from which Figure 3.8 was compiled.

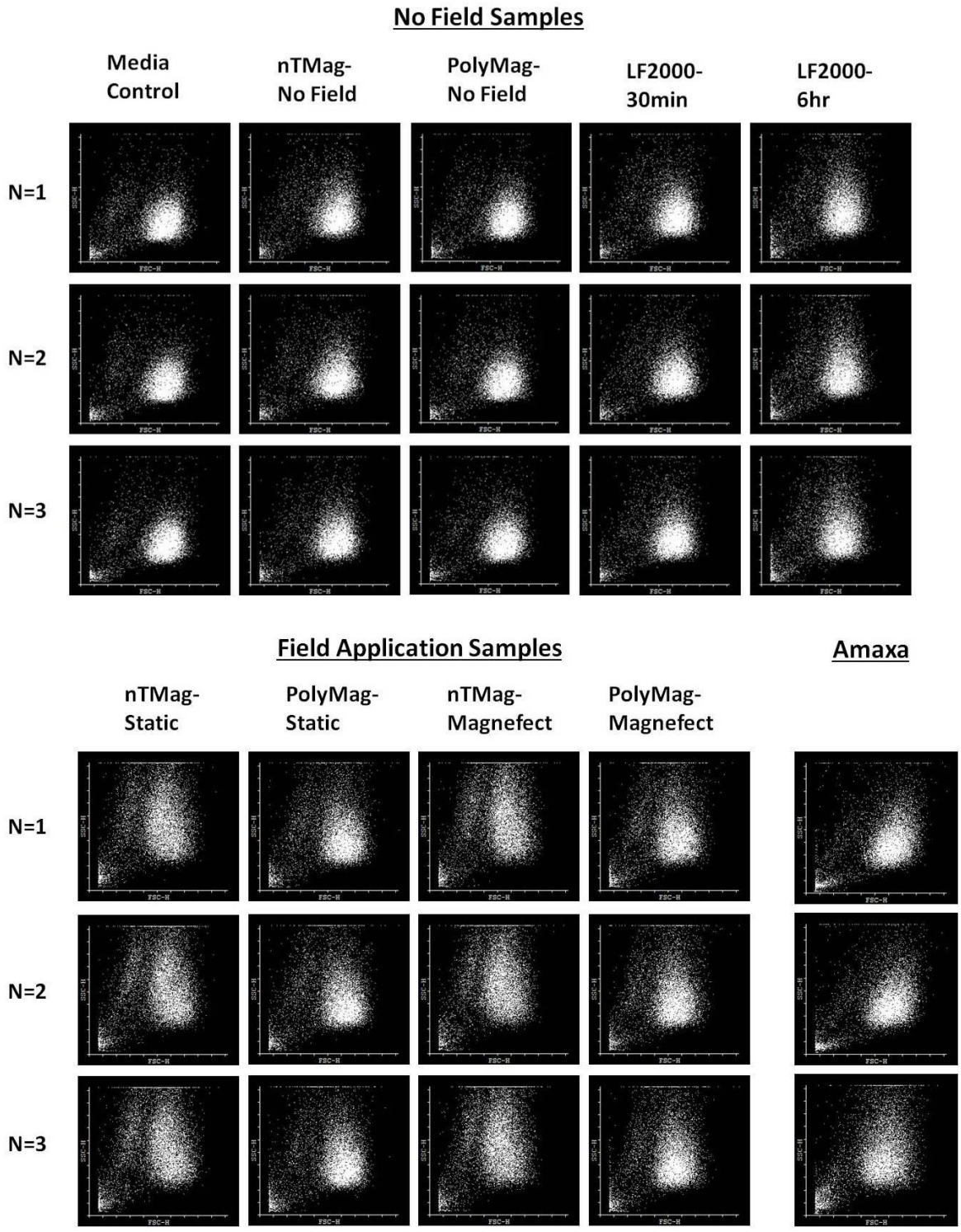


Figure 3.9: GFP expression following transfection of MG-63 cells using flow cytometry. MG-63 cells transfected with nTMag and PolyMag MNPs coated with pEGFPN1 DNA in the absence of a magnetic field, in the presence of the nanoTherics

static magnetic array and the nanoTherics magnefect-nano™ oscillating magnetic array (200 μm amplitude, 2 Hz) for 30 min, in comparison with lipid-based transfection (LF2000, for 30 min and 6 hr) and electroporation (Amaxa) methods. Data represent the mean ±SD of at least three independent experiments (N=3).

3.2.3.4 Magnetic nanoparticle toxicity and cell viability

At 48 hr post transfection, the cell viability and the corresponding toxicity of the particles for no field, static and oscillating field conditions were tested using a Cytotox-ONE Homogeneous membrane integrity assay (Promega) that measures LDH release from cells with non-intact cell membranes. These results were compared with the equivalent data for Lipofectamine and electroporation (Amaxa) using the same cell viability assay. The Amaxa cell viability data were obtained by manual cell counting in order to determine the number of cells remaining viable immediately after electroporation and 48 hr post-transfection. The percentage of transfected cells was determined by FACS analysis of the transfected cell count from the cell population that originally survived electroporation. The actual transfection efficiency of the starting population is therefore lower. Viability of cells transfected using static and oscillating magnetic field conditions with nTMag, PolyMag and Lipofectamine at 30 min were comparable, and marginally lower relative to controls (Figure 3.6).

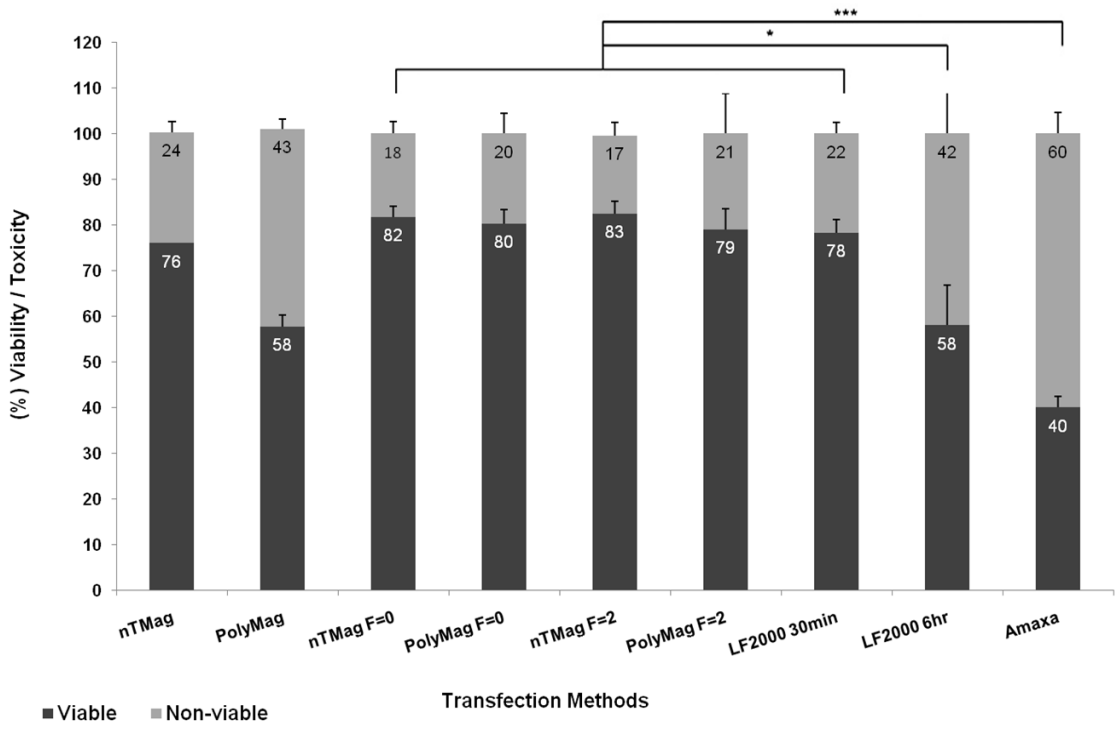


Figure 3.10: Cell viability of MG-63 cells following transfection. Bar chart showing combined average percentage for viable and non-viable cells following treatment with nTMag and PolyMag MNPs and 'no field', 'static field' (F=0) and 'magnefect-nano' (F=2Hz) transfections at 30 min, compared with Lipofectamine at 30 min and 6hr and Amaxa, at 48hrs post transfection and electroporation. n = 12 for all groups, except Amaxa (n = 8). Data represent the mean ±SD of at least three independent experiments (N=3). (Image taken from Fouriki *et al.*, 2012)

3.3 Nanomagnetic Gene Transfection for Non-Viral Gene Delivery in Mouse Embryonic Fibroblasts (NIH-3T3)

3.3.1 Objectives of the study

The objective of this work was to examine the potential of nTMag MNPs in transfecting NIH3T3 mouse embryonic fibroblasts and to investigate the effects of a novel non-viral oscillating nanomagnetic gene transfection system (magnefect-nano™) in enhancing transfection efficiency at shorter transfection times. The results obtained were compared with the equivalent from lipid mediated gene delivery and cell viability assays were performed to investigate whether the nTMag MNPs:DNA complexes had any toxic effects in the cell cultures.

3.3.2 Study design

NHI-3T3 cells were transfected using nTMag MNPs coupled with a GFP-carrying plasmid. The magnefect-nano™ system was evaluated for transfection efficiency at shorter transfection times (30 min transfection duration) and fluorescent microscopy was used to visualize GFP-expressing cells following transfection. The method was compared with lipid mediated gene delivery (cationic lipids) (6 hour transfection duration). Furthermore, cell viability studies were used to determine whether the application of the nTMag MNPs:DNA complexes and the use of static and oscillating magnetic fields had any associated toxic effects on cell viability following treatment using Cytotox-ONE cell viability assay and compared with the equivalent results from lipid mediated gene delivery.

3.3.3 Results

3.3.3.1 DNA Binding and Transfection

Binding assays demonstrated that nTMag MNPs could efficiently bind pEGFP-N1 DNA as shown in Figure 3.2 (as described in section 2.3.2). The optimal nTMag MNPs:DNA ratios used to form the transfection complexes for NIH-3T3 cells transfection were those that nearly reached particle saturation levels for both particles that demonstrated very similar binding curves (0.2 μl MNPs and 0.2 μg DNA). In addition, nTMag MNPs:DNA dose response experiments were performed as described in 2.3.3 section to obtain the optimal nTMag MNP:DNA ratios used to form the transfection complexes that confirmed findings from the nTMag MNPs:DNA binding curves. It was shown that the ratio of 0.2μl nTMag MNPs and 0.2μg pEGFP-N1 DNA was the most effective for $1x10^4$ NIH-3T3 per 96-well.

Once transfection complexes were formed and added to the cell culture, the magnet array used for static and oscillating transfections (F = 2 Hz and amplitude = 0.2 mm) shown in Figure 3.5, was placed beneath the cell culture for 30 min. In parallel, transfection with lipid mediated gene delivery (Lipofectamine 2000, LF2000) was performed as described in methods section. Following 48 hours of incubation fluorescence microscope images show a clear increase and similar GFP-expression levels between the samples transfected with nTMag MNPs exposed to magnetic fields, both static and oscillating (magnefect-nano™) for 30min, in comparison to those transfected in the absence of a magnetic field and with Lipofectamine 2000 for 30min and 6 hr (Figure 3.11).

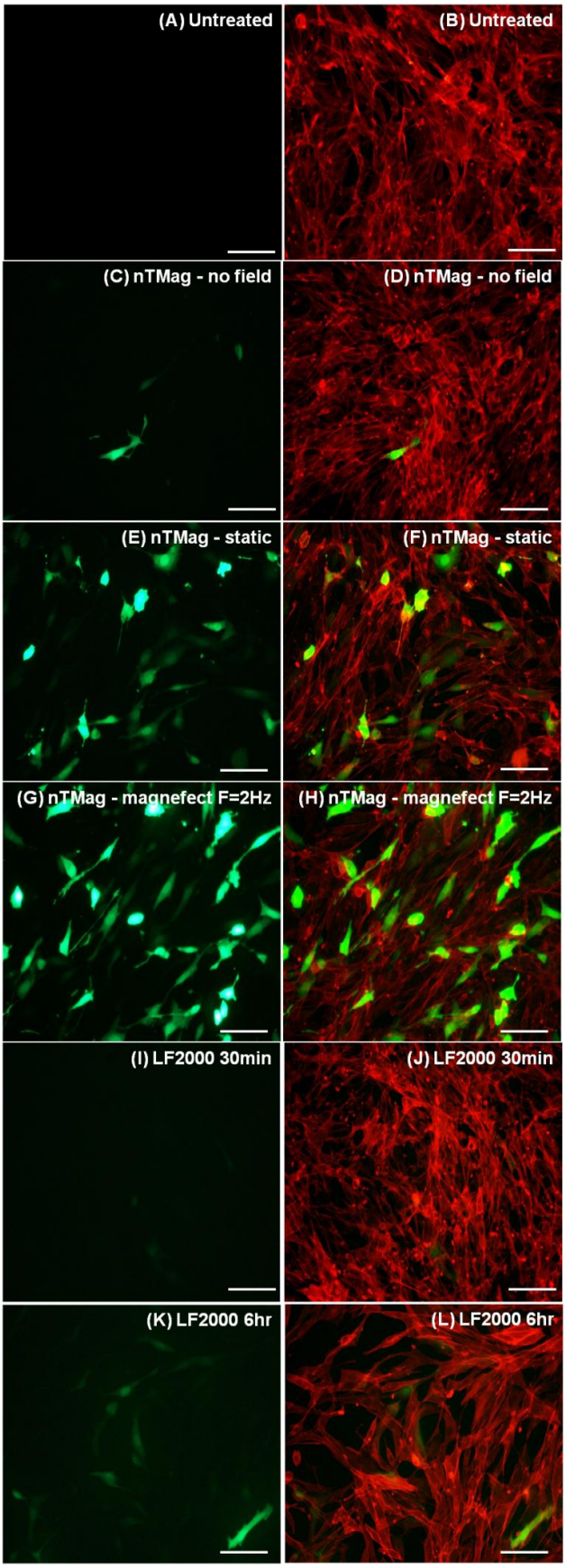

(A) Untreated
(B) Untreated
(C) nTMag - no field
(D) nTMag - no field
(E) nTMag - static
(F) nTMag - static
(G) nTMag - magnefect F=2Hz
(H) nTMag - magnefect F=2Hz
(I) LF2000 30min
(J) LF2000 30min
(K) LF2000 6hr
(L) LF2000 6hr

Figure 3.11: Fluorescent microscopy images of transfected NIH3T3 cells expressing GFP. NIH-3T3 cell were labelled with Phalloidin for actin stain of the whole cell population. **(A-B)** Untreated **(C-L)** transfected with 100 nm nTMag MNPs coated with pEGFPN1 plasmid DNA; in the absence of a magnetic field, for 30 min **(C-D)**, in the presence of a static field (nanoTherics static array) for 30 min **(E-F)** and an oscillating field (nanoTherics magnefect-nano™ array at F = 2 Hz and amplitude = 200 μm), for 30 min **(G-H)**, Lipofectamine 2000^{TM} for 30 min **(I-J)** and Lipofectamine 2000^{TM} for 6 hr **(K-L)**. Cell seeding density was 1x10^4/ 96-well, incubation period (48 hr, 37 ˚C, 5 % CO_2) post transfection and scale bar = 100μm in **(A-L)**. Data represent at least three independent experiments (N=3). GFP: green fluorescent protein; nTMag MNPs: nanoTherics nTMag magnetic nanoparticles; F: oscillation frequency. (Image taken from Fouriki et al., 2013)

3.3.3.2 Quantitative Analysis of transfection efficiency (FACS)

Quantitative analysis of transfection efficiency obtained using Fluorescence-Activated Cell Sorting (FACS) provided analogous results to those obtained via fluorescence microscopy. In the bar chart, the effect of the oscillating magnet array (*F*=2 Hz) using nTMag MNPs at 30 min was compared to "no field" and "static field" (*F*=0) conditions (at 30 min), as well as lipid-mediated gene transfection method at 30 min and 6 hr (Figure 3.12).

Some GFP expression representing transfection efficiency (2%) was observed in the presence of nTMag MNPs:DNA complexes even in the absence of a magnetic field. However, a clear increase in GFP-expressing NIH3T3 cells (25%) was demonstrated when nTMag MNPs:DNA complexes were introduced to the oscillating magnetic field

(magnefect-nano system, nanoTherics) and to a static magnetic field (22%). The increase in transfection efficiency observed between the no field and both the static and oscillating field conditions was found to be statistically significant (***p<0.001). Furthermore, the use of both the static and oscillating field conditions significantly increased transfection efficiency in comparison to Lipofectamine 2000 at 30 min (***p<0.001), but found to have analogous results to Lipofectamine 2000 at 6 hr (22%) highlighting further the efficiency of the magnetic field systems at shorter transfection times.

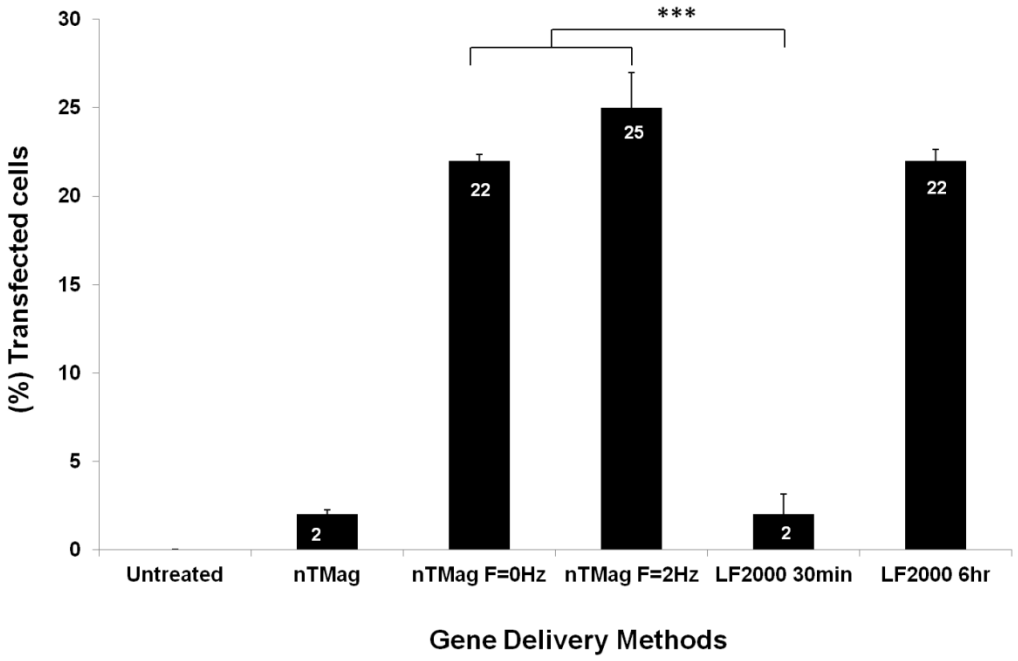


Figure 3.12: Transfection efficiency of NIH-3T3 cells. Average of FACS data from transfection efficiency of GFP-expressing NIH3T3 cells transfected with nTMag MNPs coated with pEGFPN1 DNA in the absence of a magnetic field, in the presence of the nanoTherics static magnetic array and the nanoTherics magnefect-nano™ oscillating magnetic array (200 μm amplitude, F= 2 Hz) for 30 min, in comparison to lipid-based

transfection (LF2000, for 30 min and 6 hr). During transfection cells were incubated at 37˚C and 5% CO_2. At 30min post transfection the magnets were removed and cells were incubated for 48 hr before analysis. n=6 for all samples. Data represent the mean ±SD of at least three independent experiments (N=3). GFP: Green fluorescent protein; F: oscillation frequency. (Image taken from Fouriki *et al.*, 2013)

Following Figure 3.12, summarizing the average of FACS data from transfected NIH-3T3 cells, next figure (Figure 3.13) shows the GFP expression data, from which Figure 3.12 was compiled.

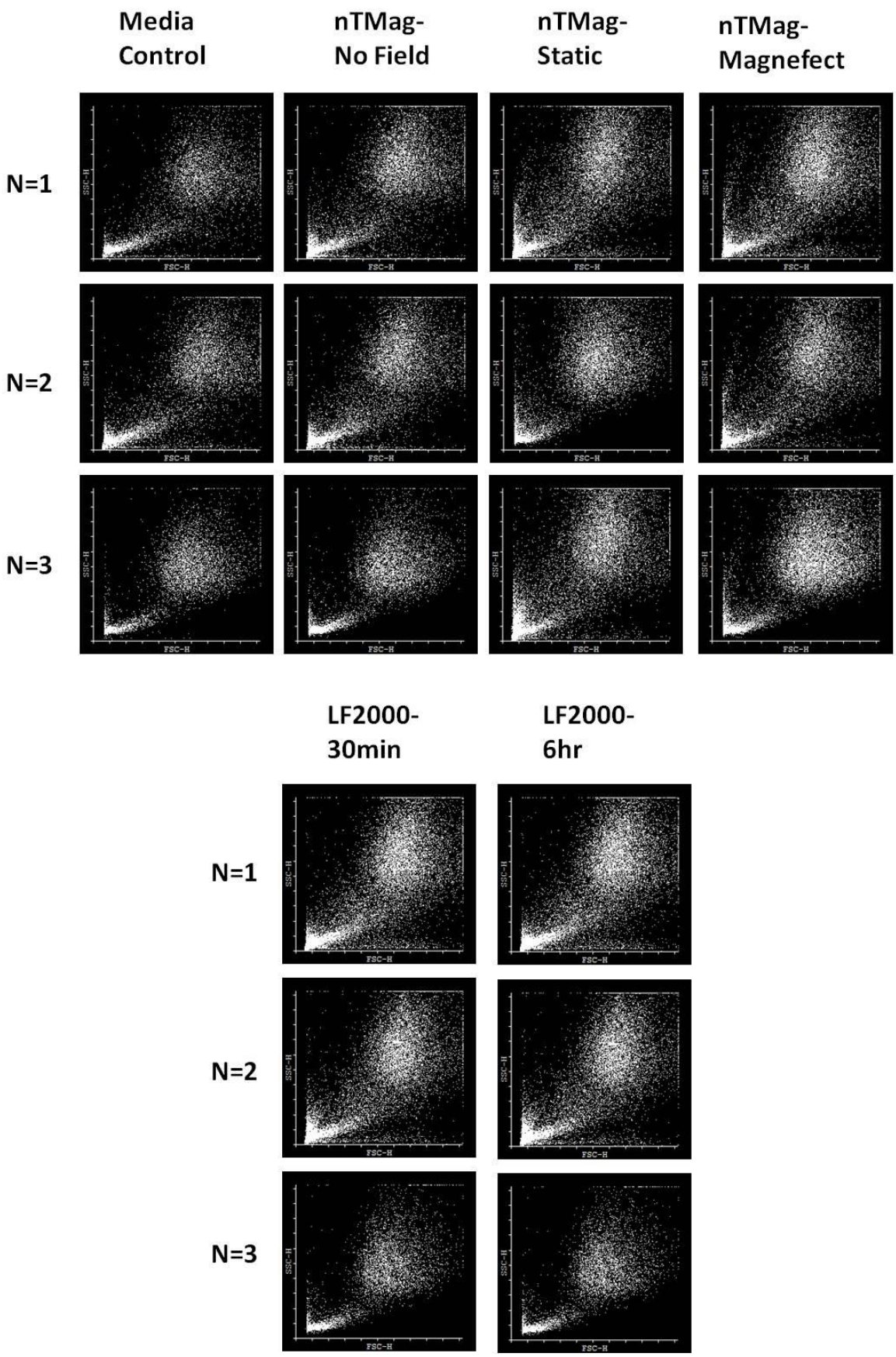

Media Control
nTMag- No Field
nTMag- Static
nTMag- Magnefect
N=1
N=2
N=3
SSC-H
FSC-H
LF2000- 30min
LF2000- 6hr
N=1
N=2
N=3
SSC-H
FSC-H

Figure 3.13: GFP expression following transfection of NIH-3T3 cells using flow cytometry. NIH3T3 cells transfected with nTMag MNPs coated with pEGFPN1 DNA in the absence of a magnetic field, in the presence of the nanoTherics static magnetic array and the nanoTherics magnefect-nano™ oscillating magnetic array (200 µm amplitude, F= 2 Hz) for 30 min, in comparison to lipid-based transfection (LF2000, for 30 min and 6 hr). During transfection cells were incubated at 37˚C and 5% CO_2. At 30min post transfection the magnets were removed and cells were incubated for 48 hr before analysis. n=6 for all samples. Data represent the mean ±SD of at least three independent experiments (N=3). GFP: Green fluorescent protein; F: oscillation frequency.

3.3.3.3 Magnetic nanoparticle toxicity and cell viability

At 48 hr post transfection, the cell viability and the corresponding toxicity of the MNPs for no field, static and oscillating field conditions were tested using a Cytotox-ONE Homogeneous membrane integrity assay that measures LDH release from cells with non-intact cell membranes. These results were compared with the equivalent data for Lipofectamine 2000 at 30 min and 6 hr, using the same cell viability assay. From the cell viability/toxicity bar chart it was demonstrated that cell viability of transfected NIH3T3 cells using nTMag MNPs at no field, static and oscillating magnetic field conditions at 30 min were very similar to the untreated controls retaining high cell viability (Figure 3.14).

Transfected NIH3T3 cells with Lipofectamine 2000 at 30 min were found analogous with all the MNP-treated and untreated samples, however, significantly lower cell viability /

higher toxicity was obtained when comparing these samples with transfected NIH3T3 cells with Lipofectamine 2000 for 6 hr (**p<0.01).

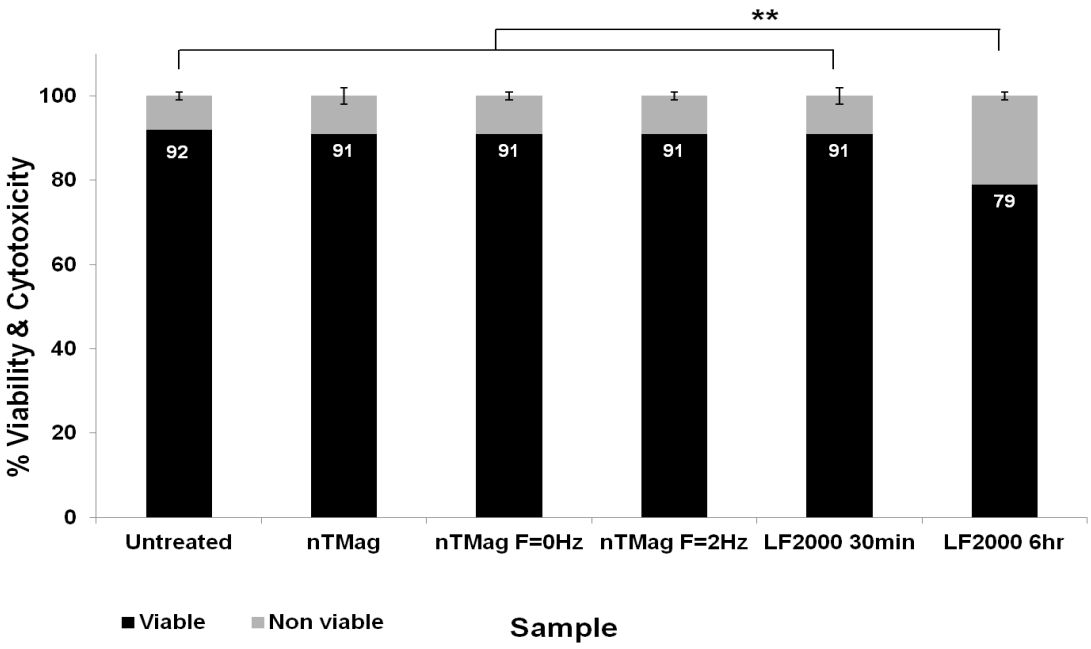


Figure 3.14: Cell viability of transfected NIH-3T3 cells. Bar chart showing combined average percentage for viable and non-viable cells following treatment with nTMag MNPs and 'no field', 'static field' (F=0Hz) and 'magnefect-nano' (F=2Hz) transfections at 30 min, compared with Lipofectamine 2000 at 30 min and 6hr, at 48hrs post transfection. n = 9 for all groups. Data represent the mean ±SD of at least three independent experiments (N=3). (Image taken from Fouriki *et al.*, 2013)

3.4 Oscillating Magnet Array-Based Nanomagnetic Gene Transfection of Human Mesenchymal Stem Cells

3.4.1 Objectives of the study

The primary objective of this work was to investigate the potential of nanomagnetic gene transfection to transfect for the first time primary human mesenchymal stem cells (hMSCs) and the effects of a novel non-viral oscillating magnet array system (magnefect-nano™) in enhancing transfection efficiency at shorter transfection times (30 min transfection duration). The nanomagnetic gene transfection method was compared with lipid mediated gene delivery and cell viability assays were performed to investigate for any potential adverse effects of the MNP:DNA complexes in the primary cell cultures. Finally, samples from both methods used were examined for their ability to retain the hMSC phenotypic characteristics and expression of hMSC surface antigens.

3.4.2 Study design

Primary hMSC cells, that are considered hard to transfect, were transfected using nTMag MNPs coupled with a GFP-carrying plasmid that were previously shown to be more effective than PolyMag MNPs. The magnefect-nano system was evaluated for transfection efficiency at shorter transfection times and fluorescent microscopy was used to visualize GFP-expressing cells following transfection. The method was compared with lipid mediated gene delivery (cationic lipids) (6 hour duration) according to the manufacturers protocol. Furthermore, cell viability studies were used to determine whether the application of the nTMag MNPs:DNA complexes had any adverse effects on cell viability following

treatment using Cytotox-ONE cell viability assay and compared with the equivalent results from lipid mediated gene delivery. Finally it was important to investigate if the phenotype of the hMSCs was altered in the level of expression of surface antigens.

3.4.3 Results

3.4.3.1 hMSCs isolation and characterization

hMSCs were isolated from human bone marrow aspirates and cultured as described in chapter 2, section 2.2.1. Before experimentation hMSCs were characterized as described in chapter 2, section 2.7.1, in order to confirm positive expression of CD44, STRO-1, CD90, CD146 and negative expression to haematopoietic markers CD14 and CD19. Cell nuclei were stained with DAPI and phase and F-actin network images were stained with fluorescent Phalloidin to facilitate visualization of typical morphology of hMSCs (Figure 3.15).

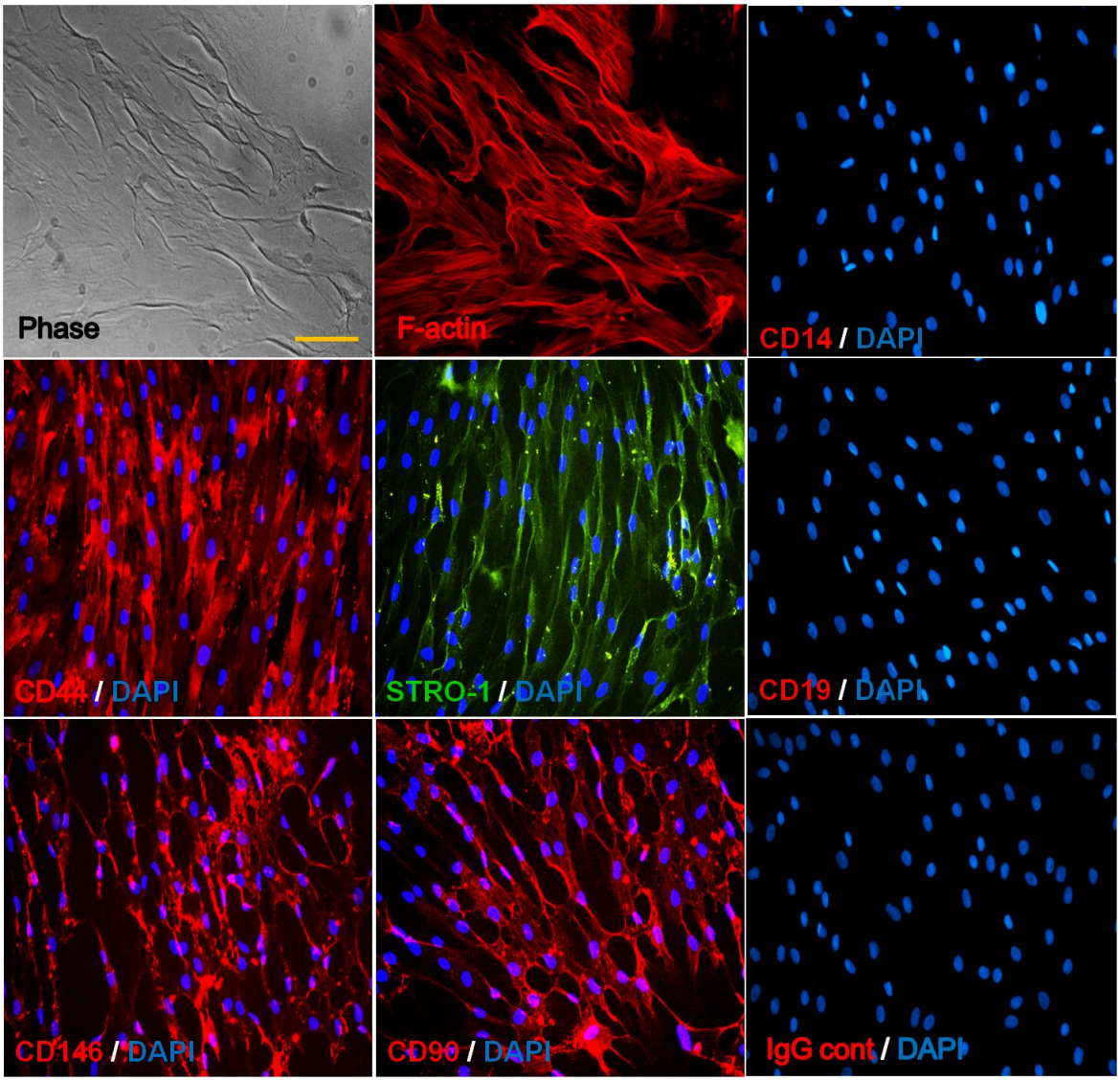


Figure 3.15: Fluorescent images of human MSC phenotypic antigenic markers. Human MSC demonstrate positive expression of CD44, STRO-1, CD90, CD146 and negative expression to haematopoietic markers CD14 and CD19. Nuclei are stained with DAPI. Phase and F-actin network images (stained with fluorescent Phalloidin) show typical morphology of hMSCs. Scale bar = 100μm. Passage 1.

3.4.3.2 DNA Binding and Transfection

Binding assays demonstrated that nTMag MNPs could efficiently bind pEGFP-N1 DNA as shown in Figure 3.2 (as described in section 2.3.2). In addition nTMag MNPs:DNA dose response experiments were performed as described in 2.3.3 section to obtain the optimal nTMag MNP:DNA ratios used to form the transfection complexes. It was shown that the ratio of 0.2μl nTMag MNPs and 0.3μg pEGFP-N1 DNA was the most effective for $5x10^3$ hESCs per 96-well .

Once transfection complexes were formed and mixed with the cell culture, static and oscillating magnet arrays (F = 2 Hz and amplitude = 0.2 mm) (shown Figure 3.5) were placed beneath the cell culture for 30 min. In parallel, transfection with lipid mediated gene delivery (Lipofectamine 2000, LF2000) was performed as described in methods section. Following 48 hours of incubation fluorescence microscope images clearly showed the highest GFP-expression levels in the samples exposed to the oscillating magnetic field (Figure 3.16). Transfection with Lipofectamine appeared quite limited.

No accurate quantification method was performed, a limitation that will be dicussed in chapter 4, due to the limited available hMSCs cell polulations. However, fluorescent microscopy images provided a qualitative estimation and a representative overview of transfection efficiency (Figure 3.16).

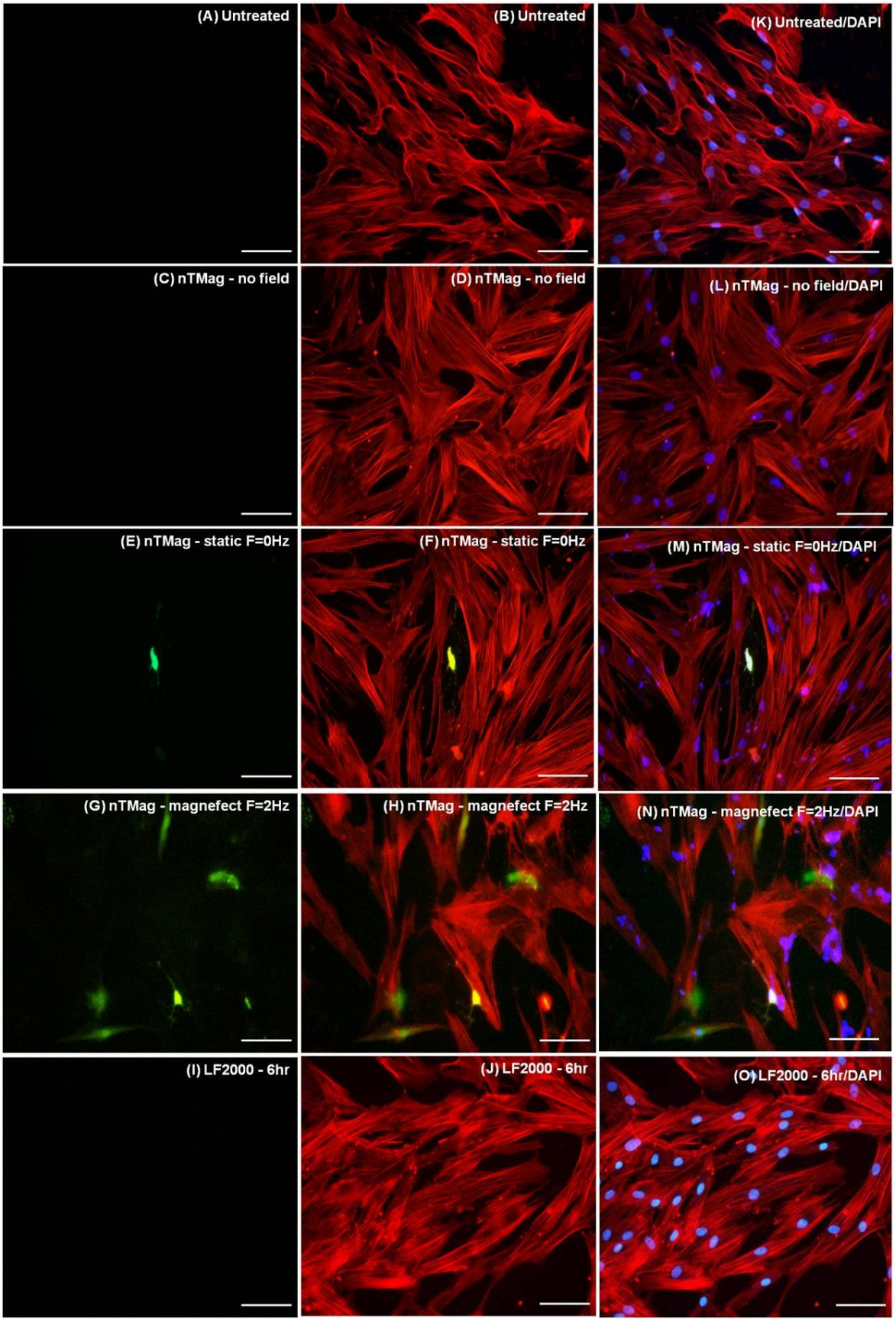

(A) Untreated
(B) Untreated
(K) Untreated/DAPI
(C) nTMag - no field
(D) nTMag - no field
(L) nTMag - no field/DAPI
(E) nTMag - static F=0Hz
(F) nTMag - static F=0Hz
(M) nTMag - static F=0Hz/DAPI
(G) nTMag - magnefect F=2Hz
(H) nTMag - magnefect F=2Hz
(N) nTMag - magnefect F=2Hz/DAPI
(I) LF2000 - 6hr
(J) LF2000 - 6hr
(O) LF2000 - 6hr/DAPI

Figure 3.16: Transfection of hMSCs. Fluorescent microscopy images of hMSCs expressing GFP and correspondingly labelled with Phalloidin for actin stain of the whole cell population. (A-B) Untreated, (C-H) transfected with 100 nm nTMag MNPs coated with pEGFPN1 plasmid DNA; in the absence of a magnetic field, for 30 min (C-D), in the presence of a static field (nanoTherics static array) for 30 min (E-F) and an oscillating field (nanoTherics magnefect-nano array at F = 2 Hz and amplitude = 200 μm), for 30 min (G-H), and Lipofectamine 2000 for 6 hr (I-J). Cell seeding density was $5x10^3$/ 96-well, incubation period 48 hr (at 37 ˚C, 5 % CO_2) post transfection and scale bar = 100μm in (A-J). hMSCs: human mesenchymal stem cells; GFP: green fluorescent protein; nTMag MNPs: nanoTherics nTMag magnetic nanoparticles; F: oscillation frequency. Data represent at least three independent experiments (N=3). (Image taken from Fouriki *et al.*, 2014)

3.4.3.3 Magnetic nanoparticle toxicity and cell viability

At 48 hr post transfection, the cell viability and the corresponding toxicity of the MNPs for no field, static and oscillating field conditions were tested using a Cytotox-ONE Homogeneous membrane integrity assay that measures LDH release from cells with non-intact cell membranes. These results were compared with the equivalent data for Lipofectamine 2000. From the cell viability/toxicity bar chart it can be seen that cell viability of transfected hMSCs using nTMag MNPs at no field, static and oscillating magnetic field conditions at 30 min were similar, and marginally lower relative to untreated controls (statistically insignificant) showing that treatment with MNPs and magnetic fields had no significant effect on cell viability (Figure 3.17).

However, significantly lower cell viability/higher toxicity was obtained when using Lipofectamine 2000 to transfect hMSCs (**p<0.01) (Figure 3.18). Finally, when comparing the three conditions ('no field', 'static field' and 'oscillating field') treated with nTMag MNPs no statistical significance in cell viability was obtained, highlighting further that the magnetic field improves transfection efficiency but does not affect cell viability.

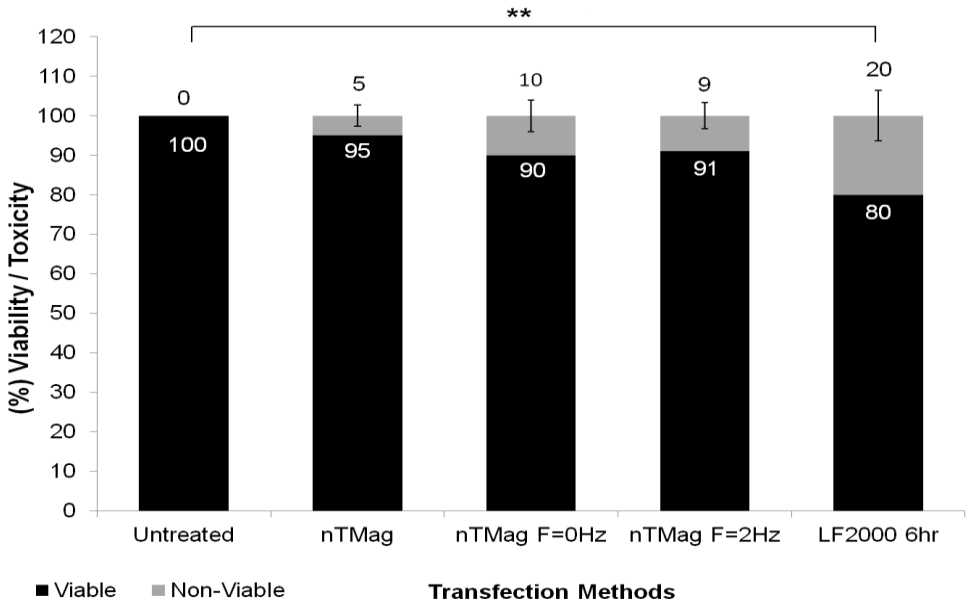


Figure 3.17: Cell viability of transfected hMSCs. Bar chart showing combined average percentage for viable and non viable cells following treatment with nTMag MNPs and 'no field' (n=9), 'static field' (F=0Hz) (n=6) and 'magnefect-nano' (F=2Hz) (n=9) transfections at 30 min, compared with Lipofectamine 2000 at 6hr (n=9), at 48hrs post transfection. Data represent the mean ±SD of at least three independent experiments (N=3). (Image taken from Fouriki *et al.*, 2014)

3.4.3.4 Immunophenotyping of transfected hMSC

Immunocytochemistry was performed in order to investigate if transfection with the magnefect-nano system and Lipofectamine 2000 has altered the hMSC multipotent phenotype (as described in section 2.7). Immunophenotyping showed the previously characterized hMSCs (Figure 3.15), retained positive expression for all four cell surface markers present on hMSCs that included antibodies directed against cell-surface molecules: CD44, STRO-1, CD90 and CD146. These results indicated that the hMSCs retained their cell-specific marker expression following treatment (Figure 3.9).

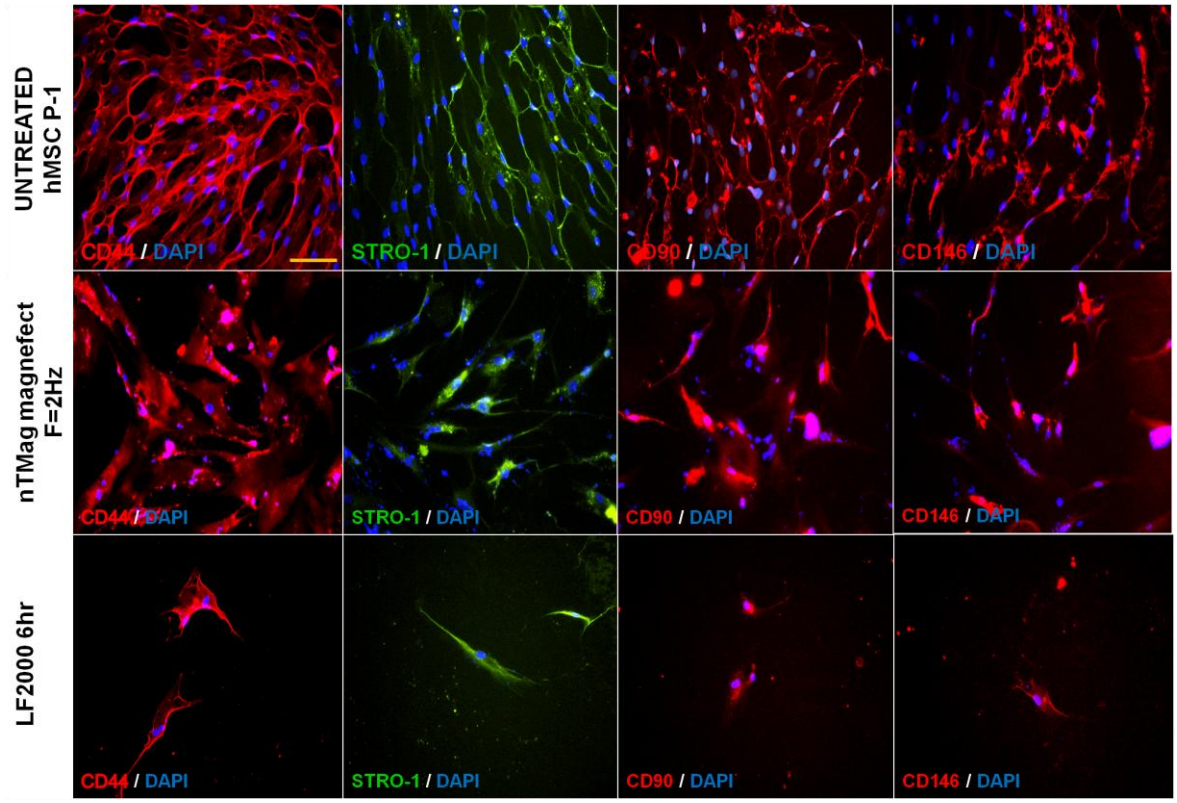


Figure 3.18: Immunophenotyping of hMSCs on isolated MSCs. hMSC transfected with the magnefect-nano system and Lipofectamine 2000 were positive for surface antigens CD44, STRO-1, CD146, and CD90 and negative for haematopoietic markers CD14 and

CD19. DAPI was used for nuclear counter staining. Scale bar, 100 μm. (Image taken from Fouriki *et al.*, 2014)

3.5 Nanomagnetic Gene Transfection for Non-Viral Gene Delivery in Human Embryonic Stem Cells

3.5.1 Objectives of the study

The aim of this work was to investigate the potential for efficient transfection of hESCs using the non-viral nanomagnetic transfection. Additionally and following 48 hrs from transfection, hESCs were evaluated for the method's impact on cell viability and capacity to retain expression of key stem cell markers for pluripotency and lineage differentiation.

3.5.2 Study design

Primary hESC cells, that have not been transfected before, were transfected using nTMag MNPs coupled with the GFP-carrying plasmid pEGFP-N1. The magnefect-nano system was evaluated for transfection efficiency at shorter transfection times and fluorescent microscopy was used to visualize GFP-expressing cells following transfection. The method was compared with lipid mediated gene delivery (cationic lipids, LF2000) (6 hour duration) according to the manufacturers protocol. Furthermore, cell viability studies were used to determine whether the application of the nTMag MNP:DNA complexes had any adverse effects on cell viability following treatment using Cytotox-ONE cell viability assay and compared with the equivalent results from lipid mediated gene delivery. Finally, it was important to investigate for potential post transfection alterations in gene expression of pluripotency associated key stem cell markers by performing semi-quantitative RT-PCR

analysis for the genes Oct-4, Nanog and hTERT, as well as for lineage differentiation markers Sox-1, ACTC1 and AFP expression.

For this purpose semi-quantitative RT-PCR was performed for the three pluripotency markers Oct-4, Nanog and *hTERT* as well as the lineage differentiation markers differentiation markers Sox-1, ACTC1 and AFP following all transfection methods.

3.5.3 Results

3.5.3.1 Determination of the magnetic field strength

In order to determine the magnetic field strength, the magnet arrays were rearranged as described in chapter 2, sections 2.3.2.1 (Figure 2.3) in the following format: In all columns magnet discs were positioned directly beneath the cell surface of the 24-well plate (at 3mm). The magnet array used for both static and oscillating transfections was mapped for its magnetic field strength as shown in Figure 3.19. The cylindrical stacks were assembled to align with 24-wel plates produced magnetic fields of ~300 mT at the cell surface.

B

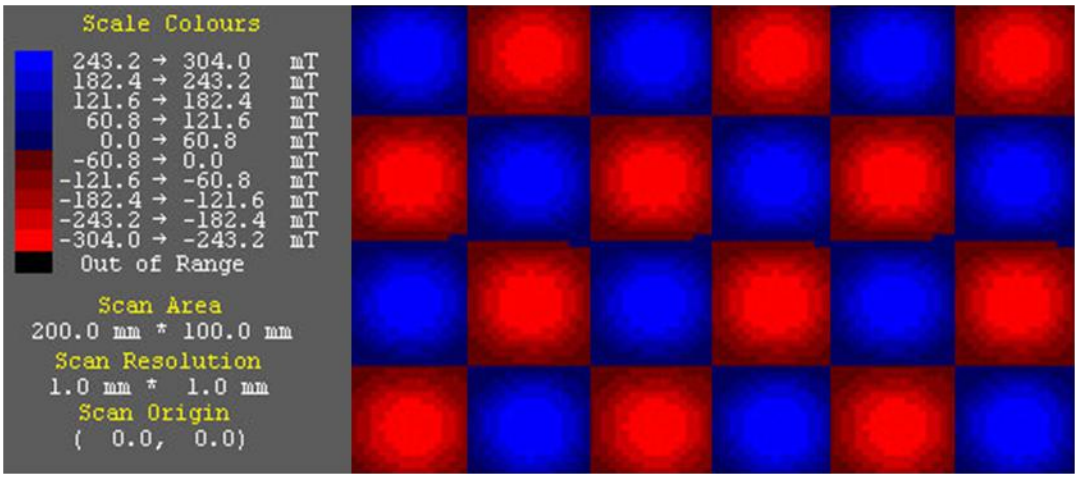


Figure 3.19: Redcliffe MagScan image of scale colours of magnetic field mapping for the nanoTherics Ltd. 24-well format magnet array. Magnet array obtained at 3 mm distance between the scanning probe and the magnet surface. At the centre of each well (at 3 mm distance) the highest magnetic field was obtained reaching 304 mT.

3.5.3.2 DNA Binding and Transfection

Binding assays demonstrated that nTMag MNPs could efficiently bind pEGFP-N1 DNA as shown in Figure 3.2 (as described in section 2.3.2). In addition nTMag MNPs:DNA dose response experiments were performed as described in 2.3.3 section to obtain the optimal nTMag MNP:DNA ratios used to form the transfection complexes. It was shown that the ratio of 1.8μl nTMag MNPs and 1.2μg pEGFP-N1 DNA was the most effective for $5x10^4$ hESCs per 24-well. For the 24-well plate format of the experiment the 24-well format magnet array was used (Figure 3.19).

Once transfection complexes were formed and added to the cell culture, static and oscillating magnet arrays (F = 2 Hz and amplitude = 0.2 mm) (shown in Figure 3.19) were placed beneath the cell culture for 30 min. In parallel, transfection with lipid mediated gene delivery (Lipofectamine 2000, LF2000) was performed as described in methods section. Following 48 hours of incubation fluorescence microscope images were obtained clearly showed the highest GFP-expression levels in the samples exposed to the oscillating magnetic field (Figure 3.20).

No accurate quantification method was performed, a limitation that will be dicussed in chapter 4, due to the limited available hMSCs cell polulations at the time of

experimentation. However, fluorescent microscopy images provided a qualitative estimation and a representative overview of transfection efficiency (Figure 3.20).

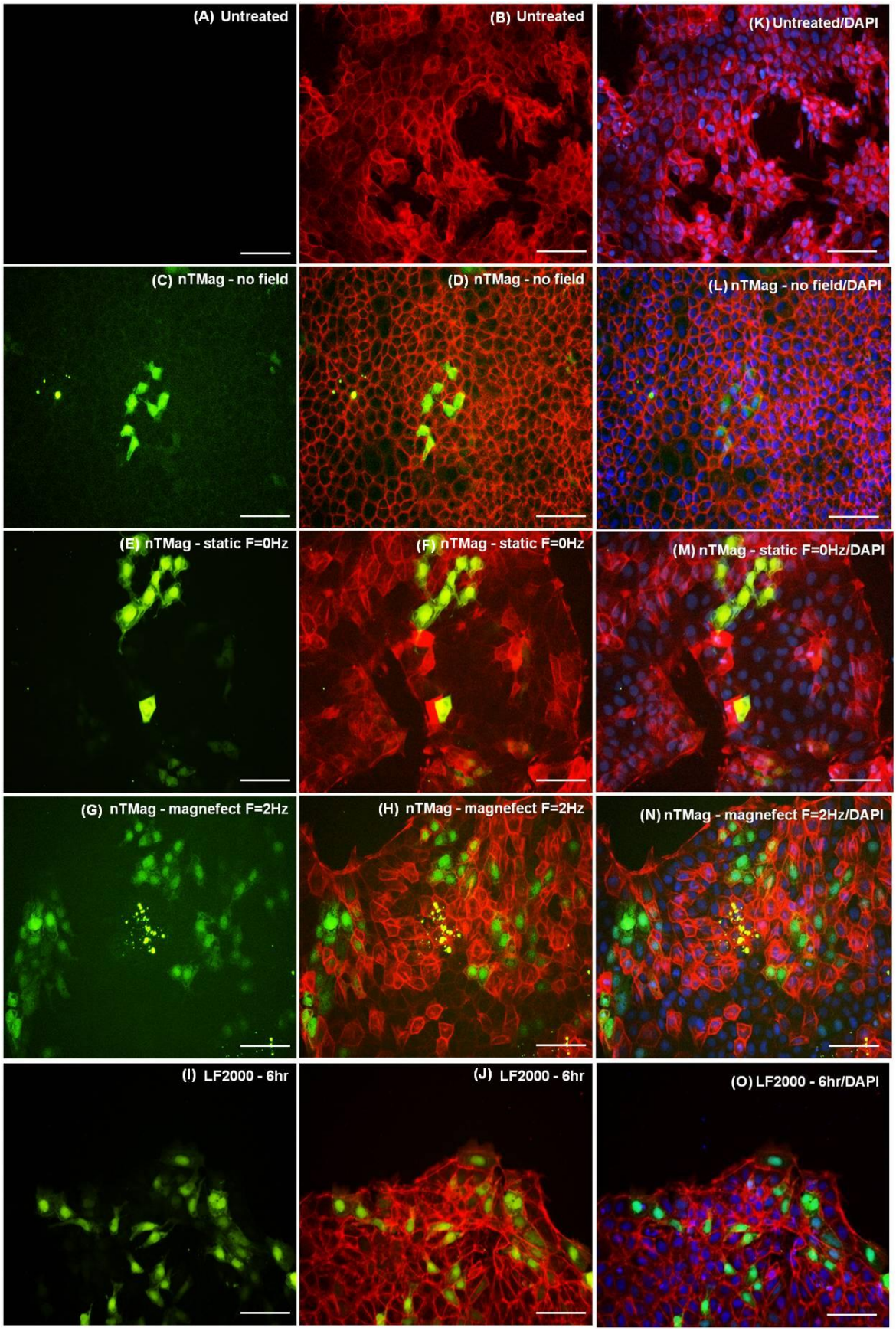

(A) Untreated
(B) Untreated
(K) Untreated/DAPI
(C) nTMag - no field
(D) nTMag - no field
(L) nTMag - no field/DAPI
(E) nTMag - static F=0Hz
(F) nTMag - static F=0Hz
(M) nTMag - static F=0Hz/DAPI
(G) nTMag - magnefect F=2Hz
(H) nTMag - magnefect F=2Hz
(N) nTMag - magnefect F=2Hz/DAPI
(I) LF2000 - 6hr
(J) LF2000 - 6hr
(O) LF2000 - 6hr/DAPI

Figure 3.20: Transfection of hESCs. (A) Fluorescent image of untreated hESCs and **(B)** labelled with Phalloidin for actin stain of the whole cell population; **(C,D)** EGFP expression and Phalloidin labeling in hESCs transfected with 100 nm nTMag MNPs coated with pEGFPN1 plasmid DNA in the absence of a magnetic field for 30 min; **(E,F)** in the presence of a static field (nanoTherics static array) for 30 min; **(G,H)** in the presence of an oscillating field (nanoTherics magnefect-nano array at f = 2 Hz and amplitude = 200 μm), for 30 min; **(I,J)** and transfected with Lipofectamine 2000^{TM} for 6 hr.
Cell seeding density was 5x10^4/ 24-well, incubation period (48 hr, 37 ˚C, 5 % CO_2) post transfection and scale bar = 100 μm. hESCs: human embryonic stem cells; GFP: green fluorescent protein; nTMag MNPs: nanoTherics nTMag magnetic nanoparticles; F: oscillation frequency. Data represent at least three independent experiments (N=3).

3.5.3.3 Magnetic nanoparticle toxicity and cell viability

At 48 hr post transfection, cell viability was tested using the Cytotox-ONE Homogeneous membrane integrity assay that measures LDH release from cells with non-intact cell membranes. Cell viability of transfected hESCs using nTMag MNPs at no field, static and oscillating magnetic field conditions at 30 min were comparable, to untreated controls (Figure 3.21).

However, significantly higher toxicity was seen when comparing untreated cells with Lipofectamine 2000 transfected hESCs (***$p<0.001$). In addition, Lipofectamine 2000 had significantly lower cell viability / higher toxicity in comparison to nTMag no field (***$p<0.001$), as well as in comparison to both static (*$p<0.05$) and oscillating magnetic

field (***p<0.001) (Figure 3.21). Finally, when comparing the three conditions (no field, static field and oscillating field) treated with nTMag MNPs no statistical significance in cell viability was obtained, further highlighting that the magnetic field improves transfection efficiency but does not significantly affect cell viability.

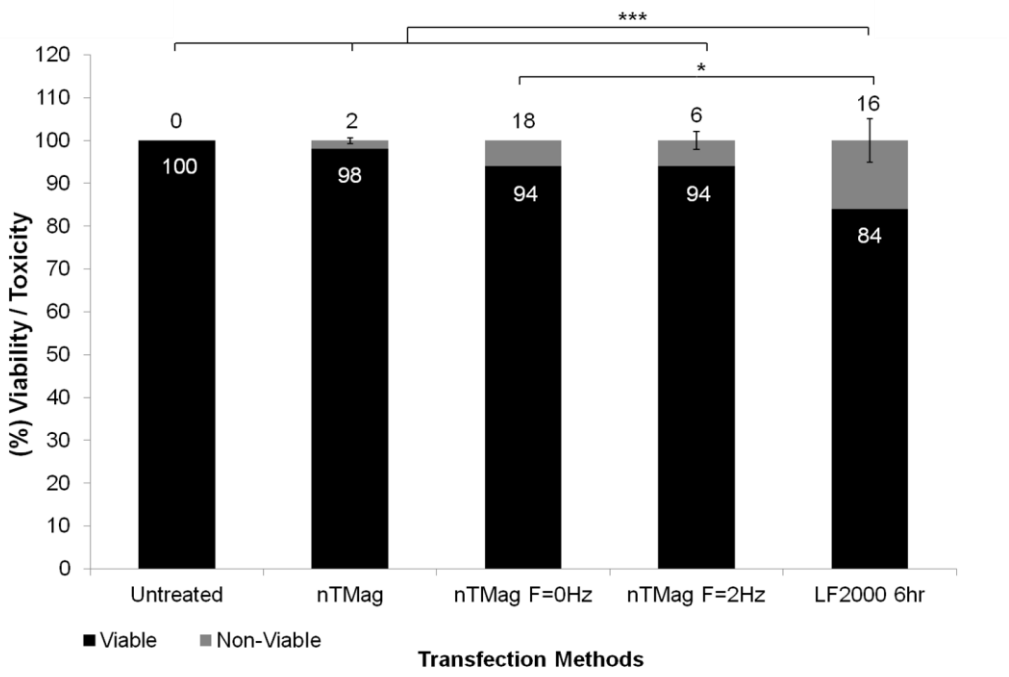


Figure 3.21 Bar chart showing combined average percentage for viable and non viable cells following treatment with nTMag MNPs and 'no field', 'static field' (F=0Hz) and 'magnefect-nano' (F=2Hz) transfections at 30 min, compared with Lipofectamine 2000 at 6hr, at 48hrs post transfection. n = 9 for all groups. Data represent the mean ±SD of at least three independent experiments (N=3).

3.5.3.4 Nanomagmetic transfection does not alter pluripotent gene expression in hESCs

To investigate whether hESCs retain expression of pluripotency and differentiation specific stem cell markers following transfection with nTMag MNPs at no field, static field, oscillating field (magnefect-nano system) as well as Lipofectamine 2000, the expression levels of hESCs pluripotent markers Oct-4 (POU5F1), h-TERT and Nanog and differentiation markers Sox-1, ACTC1 and AFP as well as the housekeeping gene b-actin (ACTB) was investigated by semi-quantitative RT-PCR. All three pluripotency markers and the housekeeping gene (Figure 3.22) as well as the differentiation markers (Figure 3.33) were detected at different intensities following all transfection methods.

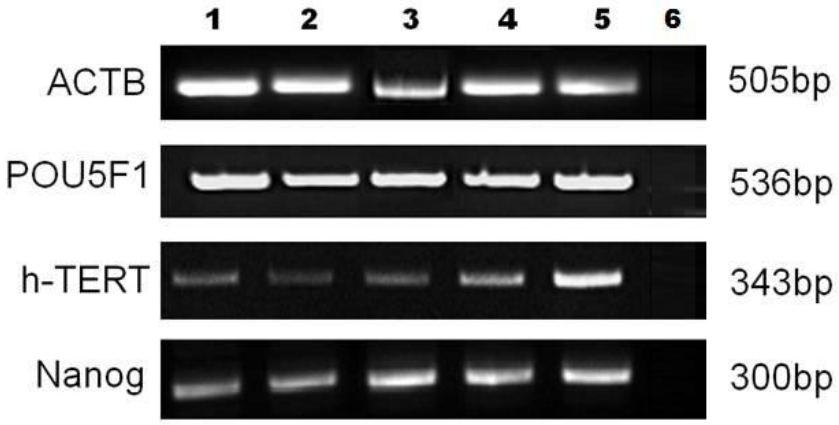


Figure 3.22 Semi-quantitative RT-PCR on transfected hESCs for pluripotency markers expression. A semi-quantitative RT-PCR analysis of hESCs POU5F1, h-TERT, Nanog pluripotency markers and the ACTB house keeping gene were assayed. Total RNA was extracted at 48hr post transfection and used for the analysis. (1) Untreated hESCs, (2-5) Transfected hESCs; with nTMag MNPs coated with pEGFPN1 plasmid DNA in the absence of a magnetic field, for 30min (2), with Lipofectamine 2000 for 6hr (3), with nTMag MNPs coated with pEGFPN1 plasmid DNA: in the presence of a static magnetic

field (*f* = 0 Hz) (4), and water control (6), and in the presence of an oscillating magnetic field (nanoTherics magnefect-nano array, *f* = 2 Hz, amplitude = 200 μm), for 30min (5).

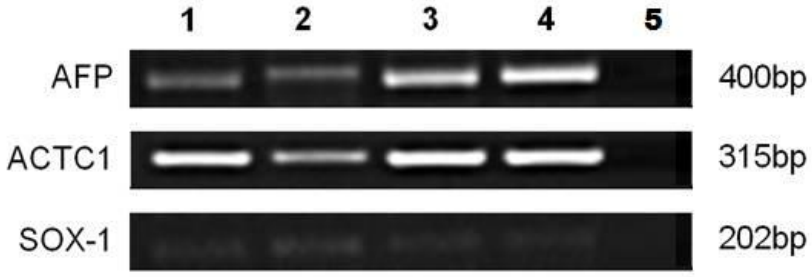


Figure 3.23 Semi-quantitative RT-PCR on transfected hESCs for differentiation markers. A semi-quantitative RT-PCR analysis of hESC differentiation markers. AFP as an endoderm marker, ACTC1 as a mesoderm marker and Sox-1 as an ectoderm marker were assayed at 48hr post transfection. (1) Untreated hESCs, (2-4) Transfected hESCs; with Lipofectamine 2000 for 6hr (2), with nTMag MNPs coated with pEGFPN1 plasmid DNA in the absence of a magnetic field, for 30min (3) and in the presence of an oscillating magnetic field (nanoTherics magnefect-nano array, F = 2 Hz, amplitude = 200 μm), for 30min (4), and water control (5).

Chapter 4

Discussion, Conclusions and Future work

4. Discussion

Gene therapy, the method used to introduce new genetic materials to hosts, is a promising tool for the study of gene function and its regulation in disease initiation and progression, for establishing various disease models, acquiring DNA-based immunization, and exploring potential therapeutic applications to various acquired or inherited diseases including neurodegenerative conditions, cardiovascular diseases, various tumours such as osteosarcoma and lung cancer, hemophilia, AIDS and asthma. As previously mentioned, the method was initially performed in 1990 in the USA for the treatment of adenosine deaminase (ADA)-deficient SCID (severe combined immune deficiency disease) patients by ex vivo retroviral-mediated introduction of the normal ADA gene into T lymphocytes (Blaese, 1993). This advancement actually led to the successful treatment of more than 30 patients since 2000 with no adverse effects related to the gene transfer technology having been observed (Ferrua et al., 2010; Ginn et al., 2013). Since then, several gene therapy success stories have been reported as described in Chapter 1 (section 1.2.2).

In recent years, there has been an increasing effort to develop new technologies for efficient gene delivery for clinical and/or scientific research purposes, which also do not adversely affect cell viability (Cho et al., 2007; Kersting et al., 2007; Lamoureux et al., 2007). Recombinant viruses, including retrovirus, lentivirus, adenovirus, adeno-associated virus, and herpes simplex virus have been used as transfection (transduction) agents and there have been numerous clinical trials with successful outcomes (Walther & Stein, 2000; Morgan et al., 2006; Cartier et al., 2009; Simonelli et al., 2010; Kalos et al., 2011; Robbins et al., 2011; Tuddenham, 2012; Ginn et al., 2013; Hammoudi et al., 2015).

However, over the past decade there has been an increasing shift towards the use of non-viral gene transfection. Several physical, chemical or hybrid approaches combining both physical and chemical characteristics have become available as described in Chapter 1 (section 1.5). Non-viral vectors are preferred due to concerns about the use of viruses, mostly related to safety, such as induced inflammatory response, target tissue specificity and insertional mutagenesis. Additionally, there are significant limitations in the plasmid packaging capacity of the different viral vectors and in the efficiency of transfecting the plasmid into suspension cell lines (Lu, 2001; Spack & Sorgi, 2001; Bhattarai et al., 2008; Mintzer & Simanek, 2009; Pickard & Chari, 2010; Naldini et al., 2011; Mingozzi and High, 2013; Kotterman & Schaffer, 2014; Cox et al., 2015).

So far, it has been demonstrated that non-viral vectors can proficiently transfect cells in culture, the most frequently used being the cationic lipid agents and cationic polymers, but still a lot of evidence is needed to obtain efficient nanomedicines. Some concerns include the strong interaction of cationic delivery vehicles with blood components, toxicity, targeting ability of the carriers to the cells of interest, the need for higher transfection efficiency compared to viral vectors.

In this study two primary aims were pursued. The first aim was the further optimization and assessment of improvements in transfection efficiency of the novel non-viral nanomagnetic gene transfection method (magnefect-nanoTM system) in hESCs and hMSCs primary cells, as well as in MG-63, NCI-H292 and NIH-3T3 cell lines in both static and oscillating conditions. The outcomes would help evaluate the applicability of the method in a variety of cell systems used for regenerative purposes. The second aim was to evaluate the efficiency of the method in comparison to the widely used lipid-mediated (Lipofectamine 2000TM) gene delivery (and selectively electroporation when possible). In parallel, cell viability and toxicity

assays were performed and morphologic and phenotypic characteristics of the cells after transfection were evaluated to determine any adverse effects of the MNP/DNA complexes' addition into the cell cultures.

4.1 Barriers of non-viral vector-mediated cell DNA delivery

The most important and difficult challenge in gene transfection is successful delivery and most efforts have been concentrated on developing safe and effective nanoparticles for systemic gene delivery In order to be efficiently used clinically, non-viral vector-mediated delivery of DNA to cells in culture has to overcome several anatomical and cellular barriers restricting the overall efficiency of gene transfer.

As it will be evident in the following sections, it has been shown by our work and the work of others that nanomagnetic transfection can significantly overcome most of these hurdles. These include vector-cell contact, transport across the cell membrane, endosomal escape in cases when the vectors are internalized within vesicles, as well as transport to and DNA delivery into the nucleus (Godbey & Mikos, 2001; Bergen et al., 2008; Zhang et al., 2012).

Vector-cell contact is principally limited by diffusion for nanoscale vectors, such as viruses and most synthetic alternatives including MNPs, and great effort has been made to facilitate more rapid vector-cell contact (Bunnell et al., 1995; Luo & Saltzman, 2000).

Crossing of the plasma membrane is typically achieved through endocytosis, the vectors being contained within endosomes, which may be subject to acidification, and trafficking to lysosomes for enzymatic digestion of the contents, preventing gene delivery. Endosomal escape can be achieved through the 'proton sponge effect' (Behr et al., 1997; Creusat et al., 2010; Lian et al., 2014; Yin et al., 2014), which is made possible through functionalizing particles with highly cationic molecules. The positive charge aids cellular uptake but also

promotes nonspecific interactions with non target cells and extracellular components such as serum proteins and extracellular matrix.

The most common way to decrease nonspecific interactions is to shield the nanoparticle surface with hydrophilic, uncharged polymers such as polyethylene glycol (PEG). Surface PEG coating sterically hinders the interaction and binding of blood components with the nanoparticle surface and prevents opsonisation and recognition by phagocytes of the RES, resulting in prolonged nanoparticle circulation in the blood. PEG coating, however, can inhibit fusion with the cell or endosomal membrane reducing the potential for particle cellular uptake and endosomal escape, decreasing transfection efficiency in case of DNA or silencing effect in case of siRNA (Zhang et al., 2012), although several strategies have been employed in an effort to handle this limitation (Walker et al., 2005; Hatakeyama et al., 2007; Hatakeyama et al., 2011).

As described analytically in Chapter 1 (section 1.7.3), in case of tumours, transport of macromolecules across the tumour endothelium is more efficient than that of normal endothelium because the tumour has a leaky and discontinuous vascular structure with poor lymphatic drainage. This is referred to as the "enhanced permeability and retention (EPR) effect" (Seymour, 1992). The tumour endothelium thus allows the penetration of macromolecules and most nanoparticles. The EPR effect can be enhanced by PEGylation because the amount of blood that circulates through the tumour is usually far less than that of the RES organs, and only the nanoparticles that are not rapidly cleared from the circulation have a chance to encounter the leaky tumour vasculature. But not all tumours are equally leaky. Thus, nanoparticles with small size (less than 30 nm) are desirable for tumours with less leaky vasculature, whereas larger nanoparticles (around 100 nm) are efficient for most tumours. The latter allow easier surface modification with PEG arranged in a brush mode.

This arrangement successfully prevents serum opsonization. Small particles are also prone to renal excretion through the glomeruli in the kidney, and their large surface curvature makes PEG shielding difficult (Schipper et al., 2009; Tseng et al., 2009; Li & Huang, 2010).

However, although PEGylation may protect nanoparticles from protein agglomeration and macrophage capture, controlling their biodistribution and tumour accumulation, the PEG coating prevents the formation of essential nonbilayer intermediates and inhibits fusion with the cell and/or the endosomal membrane, thus reducing the potential of nanoparticle cellular uptake and cargo release from endosomes, decreasing silencing or transfection efficiency (Drummont et al., 2000).

As already discussed in Chapter 1, PEI is considered one of the most effective polymer-based transfection agents and its ability to promote gene transfection in vitro and in vivo was first demonstrated in 1995 (Boussif et al., 1995). This acidification process occurs as endosomal membrane pumps import protons into the endosome. The endosomal proton pumps continue to import protons, resulting in osmotic swelling and rupture of the endosome, releasing the vectors into the cytosol. On the other hand, not much is known about how vectors are transported into the nucleus. Trafficking of PEI has been shown to be mediated by microtubule transport (Pichon et al., 2010). It is not known, however, whether this transport involved PEI within vesicles, and thus may not apply to free PEI or PEI-coated MPs (Suh et al., 2003).

4.1.1 Cells could potentially be more amenable to transfection during mitosis

In terms of DNA entrance to the nucleus for transgene expression to occur, in non-dividing cells the aforementioned process is regulated by nuclear pore complexes, and thus smaller DNA molecules gain entry more efficiently. Dividing cells on the other hand undergo nuclear

membrane breakdown (Van der Aa et al., 2006), during which nucleic acids can potentially gain entry, and that explains why proliferative populations are frequently reported to be more amenable for transfection (Bettinger et al., 2001; Brunner et al., 2000; Kim et al., 2006). As it will be discussed in further detail in section 4.6, certain cells, in our case hMSCs, are really hard to transfect using non-viral methods that result in really low transfection efficiency and reduced viability. This can be due to the fact that hMSCs have a very slow cell division cycle, which might possibly affect cellular and/or nuclear entry.

According to the currently presented data, the use of oscillating magnetic fields (magnefect-nanoTM system) for 30 min, or even shorter, transfection times at 2Hz frequency and 200 μm amplitude significantly enhanced overall transfection efficiency in comparison with absent or static magnetic fields, as well as the 6 hr treatment with lipid-based agents that demonstrated nearly complete inability to transfer hMSCs. In parallel, cell viability studies reconfirmed the very high cell viability results retained following transfection. This was not the case with Lipofectamine 2000TM where statistically significant decrease in cell viability following treatment was demonstrated. Similar results were obtained with MG-63 cells.

4.2 Nanomagnetic gene transfection

Magnetic microparticle (MP) - based gene transfection was first demonstrated in early 2000. In these experiments, magnetic microspheres with green fluorescent protein (GFP) - carrying recombinant adeno-associated virus linked to the microspheres via heparin sulfate were used. The complex was then magnetically targeted to a specific region within a culture of HeLa cells. The magnetic targeting actually enabled highly efficient uptake of the GFP gene into HeLa cells localized at the site of the applied magnetic field (Mah et al., 2000; Mah et al.,

2002). This magnetic nanoparticle-based gene transfection method was followed by the development of multiple different MNPs, coated with charged polymers to which plasmids (DNA or siRNA) are coupled for non-viral nanomagnetic gene transfection, also known as magnetofection (Scherer et al., 2002; Plank et al., 2003; Krotz et al., 2003a&b; Dobson, 2006a&b).

As already extensively discussed in Chapter 1 (section 1.8), this technique relies on a high-field, high-gradient, permanent magnetic field placed beneath a culture plate, increasing the rate of MNP/DNA complex sedimentation, and thus enhancing transfection efficiency by exploiting the magnetic properties of these particles (Plank et al., 2003). This technique has actually been successfully used to target cells carrying reporter genes, which are replaced by apoptosis genes for therapeutic applications, to tumors in vivo (Muthana et al., 2008). Although the method originally employed/used a static magnetic field, oscillation of the array of magnets beneath the culture plate, while the plate remains stationary, has been shown to enhance transfection efficiency (McBain et al., 2008b; Fouriki et al., 2010; Pickard and Chari, 2010; Jenkins et al., 2011; Lim et al., 2012; Adams et al., 2013; Subramanian et al., 2013), an observation that will be discussed in further detail in the following paragraphs, since, as already shown, it was one of the main findings of the presented work.

4.2.1 The importance of MNPs choice and the safety of magnetic fields

In order to be suitable for biomedical applications, SPIONS have to possess several key characteristics, as described in Chapter 1 (sections 1.6.2 & 1.6.3), such as biocompatibility, biodegradability, low toxicity and be physiochemically stable for storage and physiological conditions (Mahmoudi et al., 2011; Wahajuddin & Arora, 2012). For the purpose of this

thesis two commercially available SPIONs were used, nTMag and PolyMag, which actually share all the aforementioned advantageous characteristics of SPIONs for gene delivery. According to their manufacturers, they both share the same physiochemical specifications in terms of shape, size and surface coating. As already extensively described in Chapter 2 (section 2.3.3.1), they both have magnetite cores with proprietary multi-layer PEI derivative dispersed in ddH2O. Additionally, they have a diameter of approximately 100 nm and are biocompatible and biodegradable.

The use of MNPs and nanomagnetic transfection techniques compare favourably with the transfection efficiencies achieved using electroporation and other non-viral methods, such as lipid-mediated gene delivery (lipofectamine), their major advantages being high speed of transfection (up to 30 min), high cell viability post-transfection, stability and cost effectiveness. In other frequently used techniques, such as electroporation, transient pores are produced in the cell membrane, through which transfection vectors enter the cell (Leclere et al., 2005; Villemejane & Mir, 2009). However, this disruption of the cell membrane frequently results in extensive cell death and damage of the surviving cells (Gao et al., 2007; Weill et al., 2008; Villemejane & Mir, 2009).

On the contrary, MNPs do not disturb the membrane, as they make use of the natural endocytotic mechanisms of cells, and thus highly cell viability is achieved. This is a very important point when talking about their potential use in the clinical practice, as introducing dead/dying cells into a patient can have detrimental effects, such as an inflammatory response caused by the necrotic release of the cell contents (Chen et al., 2008). In addition, when cells are cultured in large scale for clinical applications, a high rate of cell attrition may add to the cost of production, especially in cases where availability of primary tissue sources is limited. Furthermore, MNPs can be a great tool for therapeutic and diagnostic (‘theragnostic’)

applications, as their multimodality enables therapeutic effects to be combined with diagnostic functionality, an example being the non-invasive imaging of MP-labelled cells (Shubayev et al., 2009). Thus, in summary, the advantages of nanomagnetic transfection using both static and oscillating magnets in vitro on various cell systems, as demonstrated by numerous studies, include: (a) low amounts of transfection complexes, (b) high cell viability, (c) high transfection efficiency, (d) little or no interference with cell proliferation and differentiation, (e) low cost (Scherer et al., 2002; Dobson et al., 2006a&b; Mykhaylyk et al., 2007; Plank & Rosenecker, 2009; Jenkins et al., 2011; Pickard et al., 2011; Sapet et al., 2011; Subramanian et al., 2013; Subramanian et al., 2017).

The ultimate goal of gene therapy and cell transplantation therapies remains the functional recovery, or at least the inhibition of disease progression. What though is of absolutely vital significance is the investigation of all possible indications of toxicity or abnormality due to MNP and magnetofection applications. No obvious effects of magnetic field application on the cells used in the currently presented experiments were observed, as discussed in the next paragraphs. The safety of magnetic field use, which is unlikely to disrupt normal cellular function via nanomagnetic activation, has been further supported by numerous other studies (Scherer et al., 2002; Huth et al., 2004; Schillinger et al., 2005; Dobson et al., 2008; Pickard & Chari, 2010; Subramanian et al., 2013).

There have, however, been a few reports were it was shown that in certain cases the application of a magnetic field affects the cells. An example of such a case was the alignment of the cytoskeletal protein F-actin when human fibroblasts were exposed to 350 mT for 30 min (Berry 2009). However, in most of those studies, prolonged field exposures (up to 60 h) were investigated and often greater field strengths were applied (up to 8T) (Smith et al., 2010). However, as a further proof of concept it would be of great interest to further explore

this by, for example, performing microarray analysis of magnetofected cells and study any possible effects on gene expression. Similar analyses could be performed for the oscillating compared to static magnetic field application, although no obvious changes were observed by any phenotypic analysis performed.

4.2.2 Oscillating magnet arrays: possible underlying mechanisms of enhanced transfection

The work presented as part of this thesis has clearly demonstrated that oscillating magnet arrays of the magnefect-nanoTM system enhanced nanomagnetic gene transfection compared to static magnetofection in NIH 3T3 mouse embryonic fibroblasts (Fouriki et al., 2010; Fouriki & Dobson, 2013), MG-63 osteoblasts (Fouriki et al., 2014), human mesenchymal stem cells (hMSCs) (Fouriki & Dobson, 2014) and human stem cells (hESCs). However, the exact mechanisms underlying this effect still remain elusive. Better understanding could help to further improve these effects either by modifying the current protocols and/or by modifying the actual nanomagnetic transfection device (magnefect-nanoTM oscillating field system).

The primary mechanism underlying magnetic - nanoparticle based gene transfection is the attraction of the magnetic particle to the magnetic field source. This results in a translational force on the particle/gene complex in the direction of the magnetic field source, which is generally placed under the culture dish. The horizontally oscillating magnet array introduces a component of lateral motion as the particles are pulled towards the field source. The extra energy and mechanical stimulation associated with this lateral motion of the particles have been shown to promote increased particle sedimentation and stimulate uptake, thus improving the transfection efficiency compared to static fields. Thus, compared to static oscillating

magnet arrays show promise for further enhancing nanomagnetic transfection and gene delivery (Dobson, 2006a&b). As discussed next, the improved transfection ability of nanomagnetic transfection using oscillating magnet arrays has been demonstrated in several cell types. However, further testing in additional cell systems would be needed.

Oscillation appears to facilitate endocytosis-mediated cellular uptake of complexes during MNP-mediated transfection (Kami et al., 2011). Additional transfection studies from our and other research groups have demonstrated similar findings using oscillating nanomagnetic transfection on mouse embryonic fibroblasts (MEFs) (Lim & Dobson, 2012), neural stem cells (Adams et al., 2013), human umbilical vein endothelial cells (HUVEC) (Lim & Dobson, 2012), rat oligodendrocyte precursor cells (Jenkins et al., 2011), rat astrocytes (Pickard & Chari, 2010), and cardiac progenitor cells (Subramanian et al., 2013).

In all these studies a frequency-dependent increase in transfection efficiency was shown, which actually demonstrated the versatility of the frequency-displacement based-nanomagnetic transfection technology as well its efficiency over a range of primary, differentiated and undifferentiated cells, and cell lines. However, the observed enhancement in transfection efficiency appears to be highly dependent on the cell type as it was shown to be quite modest in some in vitro systems, such as astrocytes (Pickard & Chari, 2010), adult cardiomyocytes (Subramanian et al., 2013), SH-SY5Y neural cell line, and primary neurons (Subramanian et al., 2017).

The reason behind frequency-displacement dependent transfection remains elusive. However, increased cytosolic Ca2+ transients and enhanced Ca2+ oscillations have been observed with increasing pacing frequency in adult mouse myocytes (Lim et al., 2000). Additionally, shear stress has been shown to induce cytosolic Ca2+ transients in different cell types (Schwarz et al., 1992; Wei et al., 2012). Subramanian et al. thus suggested that the frequency-

displacement dependent transfection they observed in cardiac cells may depend on the cytosolic Ca2+ oscillations, which is reported to interfere with the transfection and gene expression of cells (Dolmetsch et al., 1998; Preuss et al., 2000). The same could possibly apply to other cell systems where similar observations have been made.

Static nanomagnetic transfection does not alter the mechanisms of vector uptake by the cells, but significantly reduces the time taken for particles to come into contact with adherent cells and prolongs contact time as during magnetic exposure the NPs/DNA complexes gather onto the cell culture surface (Laurent et al., 2011; Plank et al., 2011). Endocytosis still remains the main suggested method for cellular uptake leading to gene delivery (Scherer et al., 2002). There are thus various theories, based on the above, that could provide a possible explanation for the enhanced transfection efficiency observed in oscillating nanomagnetic transfection.

First, an oscillating magnetic field may result in a more uniform dispersion of particles since it may simply move the MNPs laterally and so particles in a cell-free area are brought into contact with cells, thus increasing the likelihood that each particle comes into contact with a cell within a given time. In 2008, a static magnetic field model predicted that magnetic particles accumulate at the centre of a culture well, due to the radial component, potentially with an annulus of slightly greater accumulation surrounding it, due to the axial component (Furlani & Ng, 2008).

However, that was neither experienced during our experiments nor it has been reported by other groups. In addition, the applied oscillating field may lead to the distortion of MNPs or to the stimulation of the cell membrane in a way that endocytosis is more likely to occur (Dobson, 2008; Fouriki et al., 2010). It could also be possible that the applied oscillating field alters intracellular processing of MNPs by, for example, disrupting endosomal processing and facilitating endosomal escape (McBain et al., 2008). This notion is further supported by the

currently presented work on NCI-H292 cells and could possibly be linked to the very high protein production observed (Fouriki et al., 2010).

Finally, as aforementioned, the mechanical stimulation associated with the lateral motion of the particles could actually promote enhanced particle sedimentation and stimulate uptake, thus increasing the transfection efficiency compared to static fields.

Kamau and coworkers performed experiments using HeLa cells and showed that the application of a pulsating electromagnetic field (amplitude: 27 mT, field gradient: 10 T/m) greatly enhanced MP-mediated transfection. A pulsed field is thought to produce both horizontal and vertical oscillation of the MPs. The particle movements induced by these oscillations are presumed to underpin the mechanism via which the pulsed field increases transfection efficiency. These effects were observed following a 10 min exposure to magnetic fields. Taken together these findings support the idea that membrane stimulation by MPs in a dynamic magnetic field leads to enhanced cellular uptake, rather than post-uptake intracellular events. It is interesting to note that following a 5 min exposure to the magnetic field, the authors noticed that the temperature at the surface of the device had risen from 37oC to 42.5oC. Although this could be potentially damaging for the cells, the authors did not report any toxicity data (Kamau et al., 2006). During the protocols used during the work presented here, no similar heating effects were observed.

4.3 Evaluation of the magnetic field requirements for nanomagnetic gene transfection

As part of the optimization of magnetic nanoparticle - based transfection technique, it was initially necessary to understand the effects of magnetic field strength and distance on

transfection efficiency and protein production. For these experiments human lung mucoepidermoid carcinoma cells (NCI-H292) were used. In order to determine the optimal working distance between the magnet and the cell culture for the improvement of transfection efficiency in NCI-H292 cells, magnet arrays were rearranged, as previously described in detail in Chapter 2 (Section 2.3.2), to gradually increase the distance between the magnet array and the cell culture between 3 and 8 mm. The highest magnetic field reached was 101 mT. Based on the presented data of fluorescent microscopy images (Figure 3.2) it appears that the highest luciferase expression levels of NCI-H292 cells following 2 h transfection were achieved at the closest spacing between the magnet and the cells (3 mm). However, the difference in luciferase expression was not statistically significant when compared to 4 mm, 5 mm and 6 mm distances, providing scope for potentially transfecting cultured tissue explants with this technique. It was actually demonstrated by these results that there is a plateau effect beyond which transfection efficiency is not improved (Fouriki et al., 2010).

This demonstrated correlation between the magnetic field strength and transfection efficiency then formed the basis for the way the magnet arrays were assembled for the studies that followed, meaning that cell cultures were positioned in the closest proximity with the magnet array. It is of great importance to mention that regardless of the magnet distance luciferase activity using the oscillating field was statistically significantly enhanced compared to both static field and Lipofectamine 2000TM after both 2 and 6h transfection (Fouriki et al., 2010). These results were in line with earlier studies of oscillating magnetic systems where increases in luciferase activity and in transfection efficiency using GFP have been reported (Kamau et al., 2006; McBain et al., 2008; Pickard et al., 2010). It should also be noted that in our experiments the luciferase activity was not directly related to the number/ percentage of cells transfected, but rather was a proxy for protein/ enzyme production (Fouriki et al., 2010).

Furthermore, cell viability studies demonstrated unaffected cell viability levels, comparable to untreated samples, following transfection with the MNPs/DNA complexes and magnetic fields eliminating any concerns about MNP toxicity.

Data from NCI-H292 cells also revealed one of the main advantages of the method. As the forces generated on the particle/plasmid complex were in the picoNewton range, the effects on the cell membrane integrity were negligible. Based on findings from previous studies of magnetic ion channel activation (Cartmell et al., 2002; Dobson et al., 2006; Dobson et al., 2008), the primary mechanism for uptake is likely to be via increased endocytosis due to mechanical stimulation (Fouriki et al., 2010). Although this mechanical stimulation can potentially affect downstream protein production in mechanoresponsive cells, reporter constructs such as luciferase and GFP are dependent on cell entry and transcription rather than anomalous protein expression (Cartmell et al., 2002).

4.4 Nanomagnetic gene transfection for non-viral gene delivery in NIH-3T3 mouse embryonic fibroblasts (MEFs)

The potential of this transfection method was also investigated in NIH-3T3 MEFs. Based on the data presented, in this case the oscillating nanomagnetic gene transfection method had similar transfection efficiency results (25%) with the static array (22%), still slightly more improved. The increase in transfection efficiency observed between the no field, and both the static and oscillating field conditions was found to be statistically significant (***$p < 0.001$). Additionally, the use of both the static and oscillating field conditions significantly increased transfection efficiency compared to Lipofectamine 2000TM at 30 min (***$p < 0.001$), but was found to have analogous results to Lipofectamine 2000TM at 6h (22%). These results

demonstrate the efficiency of the magnetic field systems at shorter transfection times using lower reagent doses. Furthermore, during these experiments, excellent cell viability levels were retained comparable to untreated samples and significantly higher cell viability was observed compared to Lipofectamine 2000TM at 6h (Fouriki and Dobson, 2013).

An interesting finding in the course of these experiments was that Lipofectamine 2000TM-transfected cells glowed less brightly compared to cells transfected with the magnefect-nanoTM system. This observation further supports the idea suggested in the previous section and what has since been shown by other studies (McBain et al., 2008; Fouriki et al., 2010; Pickard and Chari, 2010), that protein production is enhanced in the magnefect-nanoTM system.

Taken together the presented data have shown the potential of nanomagnetic transfection to be a fast and efficient method for nonviral transfection of MEFs for use in regenerative medicine and tissue engineering research applications.

4.5 Transfection of MG-63 Osteoblasts using magnetic nanoparticles and oscillating magnetic fields

Osteosarcoma is the most common malignant bone tumour and occurs mainly in children and adolescents (Klein and Siegal, 2006; Liang et al., 2009). Conventional therapies have so far provided limited, if any, improvement and thus increasing emphasis has alternatively been given on gene therapy approaches (Cho et al., 2007; Kersting et al., 2007; Lamoureux et al., 2007). In order to examine the potential of the magnefect-nanoTM transfection system we used the human osteoblastic/osteosarcoma cell line MG-63, which has been extensively used

in various studies of bone tissue engineering, regenerative medicine and the metastatic mechanism of bone cancers (Shi et al., 2005).

The method (both static and oscillating magnetic fields) resulted in higher transfection efficiency (49% and 53% respectively) than lipid-based reagents (7%) and electroporation (21%). Cell viability was found to be relatively unaffected, mainly in comparison to 6h lipid transfection and electroporation, and cell morphology appeared normal after transfection. The highest levels of transfection (53%) were achieved using nTMag nanoparticles with an oscillating magnet array running at a frequency of 2 Hz and amplitude of 200 μm (Fouriki et al., 2014). These frequencies were significantly lower than those used for magnetic fluid hyperthermia, which was aimed at tumor ablation or induction of apoptosis (Pankhurst et al., 2003).

These methods rely on coupling high frequency fields to magnetic nanoparticles to heat the particles or mechanically disrupt the cell membrane. At the frequencies used in the current experiments, neither mechanism occurred, thus the effects on cell viability were negligible. It is also important to notice that the magnefect-nanoTM system demonstrated a 2-fold increase in transfection efficiency in 30 min compared to Lipofectamine 2000TM subjected to 6 h duration (24%). Similar was the significance of improved transfection in comparison to electroporation.

Interestingly, some transfection was seen when using the nTMag particles even in the absence of an applied field; while for both static and oscillating magnet conditions, nTMag particles appeared to provide significantly enhanced transfection efficiency than PolyMag (24% for static and 20% for oscillating). Taken together, these results showed that nanomagnetic transfection provides a fast and efficient method for non-viral gene transfection (or delivery) that outperforms both lipid-mediated gene delivery and

electroporation. They also demonstrated that nanomagnetic gene transfection has great potential for use as an effective, non-viral transfection agent for MG-63s for use in tissue engineering and cancer research applications (Fouriki et al., 2014).

4.6 Oscillating magnet array-based nanomagnetic gene transfection of human Mesenchymal Stem Cells (hMSCs)

hMSCs are non-hematopoietic stromal cells, critically important for both stem cell and gene therapy studies. They can be ideal candidates for tissue engineering and regenerative medicine applications due to their differentiation capacity and ability to regenerate multiple tissue types, such as bone, cartilage, muscle, ligament, tendon and adipose tissue (Pittenger et al., 1999; Chamberlain et al., 2007; Chen et al., 2008; Schop et al., 2009). The challenge in using hMSCs is that they are particularly hard to transfect using non-viral methods as cationic lipid reagents result in low TEs and slightly reduced viability, while electroporation results in relatively low cell viability. In addition, hMSCs have a very slow cell division cycle, which might affect cellular and/or nuclear entry. As part of this study, the potential of nanomagnetic gene transfection in the presence of oscillating magnetic fields for hMSCs transfection was explored.

It was shown that the use of the magnefect-nanoTM system for 30 min transfection at 2 Hz frequency and 200 μm amplitude significantly enhanced overall transfection efficiency in comparison with absent or static magnetic fields, as well as the 6 h treatment with lipid-based agents that demonstrated nearly complete inability to transfect hMSCs. In parallel, membrane integrity assays determined that treatment with MNPs and oscillating or static magnetic fields

retained high cell viability comparable with untreated hMSCs following transfection. This was not however the case with Lipofectamine 2000TM-treated samples which were characterized by statistically significant decrease in cell viability and increased toxicity following treatment (Fouriki & Dobson, 2014).

hMSCs divide multiple times and retain their multipotent differentiation characteristics in culture. The fact that hMSCs retained their phenotypic characteristics following treatment was confirmed by the positive expression of four cell-surface specific markers present on hMSCs: CD44, CD90, STRO-1, and CD146 (Fouriki & Dobson, 2014). This was further confirmed by the negative expression of hematopoietic cell surface markers CD14 (present on leukocytes) and CD19 (present on B-lymphocytes) (Chamberlain et al., 2007; Chen et al., 2008), which actually demonstrated there was no hematopoietic cell contamination (Fouriki & Dobson, 2014). Additional negative markers include CD45, CD34 (Chamberlain et al., 2007; Chen et al., 2008).

An interesting observation made during these experiments was the significantly enhanced accumulation of DAPI staining in the oscillating magnet samples compared to the static ones. Although this could be a random finding, it could also have another explanation. It is not clear from the staining whether this increased accumulation is inside or outside of the cell. If this is indeed the case, then this could be attributed to compromised ability of the plasmid to enter the nucleus or the cell respectively. Although being a cell entry problem is still possible, it is highly unlikely, as there is no reference suggesting that hMSCs cell membrane could not be permeable with this method. Being a nuclear entry problem is most likely, also based on the fact that hMSCs are slow dividing cells and similar to all non-dividing cells they are very hard to transfect based on the fact they have a very slow division cycle and as already discussed in section 4.1.1 cells are more amenable to transfection during mitosis. However,

further experiments, including live monitoring and electron microscopy would be needed in order to verify it is a nuclear entry problem.

Another observation during the hMSCs experiments was the presence of protein RAFTS, shown as pigmented dots in untreated hMCs characterization. This could be in support to the theory that genes are highly expressed in particular places, known as protein RAFTS, which are a group of organized protein (Lucero & Robbins, 2004).

The currently presented work on hMSCs was limited by the fact that FACS analysis was not performed due to low number of cells. Samples were thus assessed by microscopy and manual counting was performed. These data, although mostly qualitative, are in agreement with and support the rest of the data pointing towards the effectiveness of oscillating magnetofection in hMSCs transfection. Undoubtedly, future FACS experiments on these cells are needed to further verify the findings presented here and provide a more accurate quantification of transfection efficiency.

Taken together, these data demonstrate that nanomagnetic transfection provides a fast and efficient method for non-viral transfection of hMSCs and could be of great potential as an effective, non-viral transfection system for this type of cells.

4.7 Nanomagnetic Gene Transfection for Non-Viral Gene Delivery in Human Embryonic Stem Cells (hESCs)

This study represented the first demonstration of nanomagnetic transfection of hESCs using both static and oscillating magnet arrays. In recent studies cationic lipids have been used to transfect ESCs with both DNA and siRNA, however, transfection efficiencies have been relatively low and toxicity has been a great concern [Ma et al., 2011]. Fluorescent microscope

images obtained 48 hr post pEGFP-N1/MNP complex introduction into the cell culture, showed that hESCs were efficiently transfected and visual inspection of the image indicated that magnet arrays oscillating at 2 Hz appeared to enhance transfection when compared to cationic lipids, though this result should be considered preliminary.

Cell viability of hESCs following treatment with magnetic nanoparticles and both static and oscillating nanomagnetic transfection appeared unaffected relative to untreated controls. Significantly higher toxicity was observed when comparing untreated cells with Lipofectamine 2000TM – transfected hESCs (***$p < 0.001$); while Lipofectamine 2000TM had significantly lower cell viability/higher toxicity in comparison to nTMag no field (***$p < 0.001$), as well as in comparison to both static (*$p < 0.05$) and oscillating magnetic field (***$p < 0.001$). Finally, no statistical significance in cell viability was observed when comparing the three conditions (no field, static field and oscillating field), further highlighting that the magnetic field improves transfection efficiency but does not significantly affect cell viability.

Furthermore, following transfection, the expression of the specific transcriptional factors Oct-4 and Nanog (Cruz et al., 2003) and h-TERT, as a confirmation of high telomerase activity (Wobus & Boheler, 2005; Mathew et al., 2010), indicated that the cells retained expression of specific pluripotency markers. Analysis of the expression of Sox-1 (ectodermal layer marker) (Wobus & Boheler, 2005), ACTC1 (mesodermal layer) (Abeyta et al, 2004) and AFP (endodermal layer) (Osafune et al., 2008) also demonstrated that the cells expressed specific differentiation markers from all three somatic germ layers. However, the data are preliminary and reconfirmation is required, including the elimination of any possible experimental errors.

The data presented here provides some preliminary evidence that nanomagnetic transfection provides a rapid and efficient method for non-viral transfection of hESCs. In 30 min, the technique achieved transfection efficiencies similar to those obtained with a 6 hr treatment using lipid-based reagents, with significantly higher cell viability. Additionally, preliminary results have indicated that stem cell morphology, pluripotency and differentiation potential remained unaffected post-transfection. qPCR results for markers of multipotency demonstrated that these are expressed post-transfection. However, these experiments were carried out using extracted RNA from the whole cell population (transfected and non-transfected cells) and one could possibly claim that expression of these transcription factors was actually observed in the non-transfected cells. Further experiments, during which cells will be sorted by FACS prior to gene expression analysis, will provide a definite confirmation on whether this is the case or not.

Though this study reports our initial results of hESC transfection, oscillating array-based nanomagnetic gene transfection efficiency using magneto-mechanically stimulated particle/DNA (or siRNA) uptake has the potential to be improved further by systematically investigating a wider range of frequencies and amplitudes (Jenkins et al., 2011).

4.8 Summary of thesis findings and conclusion

Based on the data presented in this thesis, nanomagnetic transfection provides a fast and efficient method and has significant potential for use as an effective, non-viral transfection agent for a variety of primary human stem cells and different cell lines. Taken together these results exhibit its diverse use and potential in regenerative medicine and tissue engineering research applications.

Gene transfection and transient GFP expression was examined. It is understood that stable gene expression is governed by different requirements and mechanisms which need to be further investigated. It was shown that the use of oscillating field surpasses transfection efficiency levels compared to the static one; this difference being statistically significant in most cases. The magnet distance was also examined and the threshold at which transfection efficiency increased and marked protein production was stimulated was identified. Compared to other methods, magnetofection is time and reagent efficient, can potentiate the efficacy of a given vector and allows magnetic-field guided targeting in vivo. The principal advantage of the MNP-based method was shown to be the rapid sedimentation of the MNP/gene complex onto the target area which significantly reduces both time and dose of vector for efficient transfection. The application could be used in gene therapy for the nucleic acid delivery itself as the heart of the therapeutic strategy, in basic research and in commercial scale biotechnology approaches where nucleic acid/ gene delivery only serves as a tool for answering scientific questions. Importantly, cell viability was excellent throughout in all experiments with different types of cells.

In conclusion nanomagnetic gene transfection provides efficient non-viral delivery of MNP/DNA complexes into a wide range of cells using static and oscillating magnet arrays. The technique consistently surpasses transfection efficiency levels of the best cationic lipid agents at shorter transfection times and lower reagent doses. Cell viability and morphology of transfected cells remained unaffected. In case of primary stem cell use, hMSCs surface marker expression of CD44, STRO-1, CD90 and CD146 was retained post transfection indicating retention of phenotypic characteristics post transfection, and preliminary experiments demonstrated that hESCs retained expression of certain pluripotency (Oct-4, Nanog and hTERT) and differentiation (Sox-1, ACTC1 and AFP) markers.

4.9 Limitations of the study

A part of the work presented here is somehow limited by the fact it was carried out in cell lines. This is because cell lines possess an altered morphology compared with the corresponding primary cells. Cell lines are highly homogeneous and exhibit high survival and proliferation rates, in addition to resistance to adverse stimuli, such as cell death signals. Thus, their properties may not represent biological variations that exist in vivo, mainly in terms of cell proliferation and differentiation. Most importantly, cell lines allow for continuous passage which, if carried out without robust quality control, increases the risk of chronic contamination by mycoplasma, which can in turn alter cell structure, metabolism and growth, thus affecting the interpretation of the data (Freshney 2002). These concerns combined with the risk of cellular aneuploidy, can make cell lines a relatively poor model for toxicity testing (Freshney 2002; Hughes et al., 2007), which is currently an important issue in nanotechnology.

In addition, the proposed method in the current study was not tested enough in vivo. As already discussed in Chapter 1 (section 1.8.2), small animal studies with nanomagnetic transfection with either static or oscillating magnets have already shown great potential (Pulfer et al., 1999; Alexiou et al., 2000; Goodwin et al., 2001; Krotz de Wit et al., 2003; Schillinger et al., 2005; Muthana et al., 2008; Brett et al., 2016). However, magnetic targeting in larger animals including humans is more challenging. This is mainly due to the fact that sites of interest are further away from the magnet source and are considerably more difficult to target. As the magnetic field strength and gradient rapidly decay when the magnet source is

not close enough to the target site, a high-gradient which is necessary for capturing the MNP/DNA complexes cannot be accomplished (Dobson, 2006).

Although it is up to the experimental objective whether stable or transient expression is needed, in regards to stable transfection, one should be cautious and the whole process needs to be well controlled in order to avoid insertional mutagenesis and switching on/off a gene via an activated oncogene or tumour suppressor gene respectively.

4.10 Future perspectives

Theoretically, an ideal gene therapy vector could combine characteristics of both viral and non-viral systems in order to meet the many gene delivery challenges. Several properties of these systems would need to be brought together as the vector has to be efficient, specifically targeted, biodegradable or easy to remove, stable in the bloodstream and non-toxic (Munoz et al., 2012). In addition, it is very important that the vector is safe and initiates no inflammatory or immunogenic response, and that is it is able to protect genetic materials against degradation during transport and be stable for storage (Kay et al., 2009; Grigsby and Leong, 2010; Kay et al., 2011). We have shown that in the cell systems studied, the method used had most of these characteristics, and similar results have been demonstrated by studies on other various cells. However, the method could be further ameliorated in order to enhance transfection efficiency even further.

A way to achieve that would include adaptations in the cell culture conditions. It was recently shown that transfection efficiency in HUVEC cells using both static and oscillating magnet systems was dramatically increased by pre-incubating the cells in serum-free conditions 2 h prior to transfection using nTMag particles. It was suggested that this could be due to the

dissociation of complexes in the presence of serum suggesting weak binding of plasmid to nanoparticles (Lim & Dobson, 2012). The same was observed in HeLa, KB and 11293 cell lines using cationic PEI-polyplexes (Liu & Zhang, 2011; Lim and Dobson, 2012). This could be due to better DNA incorporation into polyplexes or onto nTMag particles in the absence of serum. The effects of serum starvation could possibly be advantageous for other cell systems. In a recent study, where the same method was used in human prenatal cardiac progenitor cells and adult cardiomyocytes, it was shown that transfection efficiency can be enhanced by coating the wells with collagen I. Oscillating magnetofection, when used for an adherent cell transfection, then seeding/ adhering parameters are key for the technique and it is likely that the increased efficiency observed in cells seeded onto rat tail collagen I was due to efficient adherence as collagen type-I is one of the fibrous proteins within extracellular matrix and, being a structural protein, it is highly biocompatible. In the cardiomyocytes, this led to increased adhesion and improved transfection efficiency with the oscillating system (Subramanian et al., 2013). Cell adhesion assays could thus possibly be used to further improve transfection efficiency. In the same study, as well as in subsequent ones on neural cells, the Neuromag MNPs were used, which was shown to also further enhance transfection efficiency. Although, this system was initially proposed to target neural cells, it was demonstrated to efficiently transfect cardiac progenitor cells and cardiomyocytes (Subramanian et al., 2013; Subramanian et al., 2017). It would be interesting to study in the future the applicability of this system on the cell systems used in the current study.

Further work could also be performed towards the optimization of the delivery of therapeutic gene sequences. Importantly, nanomagnetic gene transfection using mechanically stimulated particle/DNA uptake may be further improved by systematically investigating a wide range

of oscillation frequencies and amplitudes, as part of the optimization for each cell type. Additionally, although the method used is based on endocytic mechanisms thus allowing the uptake of large plasmids, further optimization could still be beneficial.

For example, transfection efficiency could possibly be further enhanced by minimizing the overall size of the plasmids. For this the plasmids could be engineered to produce constructs containing only the therapeutic gene sequences and the elements necessary for transcription. Furthermore, future studies could focus on identifying physiochemical parameters that may allow for the attachment and the delivery of larger plasmids and/or higher copy numbers. To achieve that, biologists should work in collaboration with material scientists and need to understand in detail the chemical interactions occurring at the organic-inorganic hybrid interface of magnetic particles and biomolecules. An example of that is the understanding of the different association of large and small plasmids with the particle surface and the implication this has on DNA attachment and intracellular release.

Nanomagnetic transfection optimization is also influenced by the properties of the MNPs used. The attractive force is directly proportional to the volume of magnetic material in the particle, magnetic field strength/ gradient of the magnet array and magnetic properties (susceptibility) of the particle (Dobson, 2006). While it is true that increases in the magnetic content (volume of magnetic material) of the particles increases the magnetic force, generally the particles used for these studies are iron oxide/ polymer composite particles. Increasing the hydrodynamic diameter is not always associated with a proportional increase in magnetic iron oxide as the polymer represents the bulk of the particle's volume. Different sized nanoparticles have been demonstrated to use different receptor-mediated endocytosis mechanisms to enter the cells. For example, it was shown that 100 nm MNPs were endocytosed through caveolae-mediated endocytosis (Lim et al., 2012), 200 nm MNPs

through clathrin- or caveolae-mediated endocytosis (Huth et al., 2004) and 300 nm MNPs through micropinocytosis and clathrin-mediated endocytosis (Pickard et al., 2011). Different entry mechanisms may be associated with increased or decreased transfection efficiency. Additionally, suitable biocompatible and biodegradable polymer coating/matrices are also important for enhancing transfection, alongside size and magnetic properties of the nanoparticles (Kami et al., 2011).

Another field where non-viral magnetic nanoparticle based transfection has been successfully used is in the generation of iPS cells. In 2011, Lee and coworkers demonstrated that nanofection-mediated iPS cells exhibited ES cell-like characteristics, including expression of endogenous pluripotency genes, differentiation of three germ layer lineages, and formation of teratomas (Lee et al., 2011). Our method could potentially be tried to further enhance efficiency of virus-free iPS cells generation, something very crucial for clinical applications in the field of regenerative medicine. Additionally, radioactive labeling of DNA could be used to determine more accurately the binding capacity of MNPs. It has been demonstrated by the experiments presented as part of this thesis that the method has significant potential for use as an effective, non-viral transfection agent for hMSCs, as well as for multiple primary cells and cell lines. These results show its diverse use and potential in regenerative medicine and tissue engineering research and applications.

One major finding presented here, which should be further explored in the near future, is the always enhanced protein production under the magnetic field (both static and oscillating), which was first observed in NCI-H292 cells (Fouriki et al., 2010). Further experiments should help uncover the mechanisms behind this upregulation, which could be of high significance and applicability in future translational assays.

Finally, although it currently appears there is a lot of promising evidence for the future use of these systems for gene therapy, there are still some extremely difficult cases which remain a challenge. The lateral motion of the MNP/gene complex, which is primarily perpendicular to the translational force exerted on the MNPs by the field gradient, may prove useful for promoting efficiency in such cases, including the penetration of the mucus lining in the lung and delivery of the therapeutic genes for cystic fibrosis (Ferrati et al., 2004; McBain et al., 2008). As already discussed in Chapter 1, there is no available cure for cystic fibrosis yet and it is considered a good candidate for gene therapy (Mason & Dunhill, 2008). Studies addressing the issue using both viral and non-viral vectors have not been successful (Griesenbach et al., 2006; Flotte, 2007; Mueller et al., 2008; Mimoto et al., 2010) and the same was the case in a very preliminary experiment using our method. However, since this method keeps overpassing most of the barriers for successful and efficient transfection, it is possible that with the right adaptations it will also prove successful in this case.

Chapter 5

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Appendix 1: Article published by Nanomedicine.

Fouriki A, Dobson J

Oscillating magnet array-based nanomagnetic gene transfection of human mesenchymal stem cells.

Nanomedicine, 2014, 9(7): 989-97. doi: 10.2217/nnm.13.74. Epub 2013 Jul 31.

Appendix 2: Article published by Journal of Tissue Engineering and Regenerative Medicine.

Fouriki A, Clements M, Farrow N, Dobson J

Efficient transfection of MG-63 osteoblasts using magnetic nanoparticles and oscillating magnetic fields.

Journal of Tissue Engineering and Regenerative Medicine, 2014, 8(3): 169-75. doi: 10.1002/term.1508. Epub 2012 Apr 12.

Appendix 3: Article published by Materials.

Fouriki A, Dobson J

Nanomagnetic gene transfection for non-viral gene delivery in NIH 3T3 mouse embryonic fibroblasts.

Materials, 2013, 6: 255-264. doi: 10.3390/ma6010255.

Appendix 4: Article published by Nano reviews.

Fouriki A, Farrow N, Clements M, Dobson J

Evaluation of the magnetic field requirements for nanomagnetic gene transfection. Nano Reviews, 2010, 1. doi: 10.3402/nano.v1i0.5167. Epub 2010 Jul 9.

Appendix 5: Book Chapter published by Francis & Taylor CRC Press.

Book Title: Magnetic Nanoparticles: From Fabrication to Clinical Applications

Editor: Nguyen TK Thanh, University College London, UK

Published: February 01, 2012 by CRC Press – 616 pages

Book Chapter Contribution:

Chapter 12 - Nanomagnetic gene transfection

Fouriki A, Dobson J